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Susceptibility of Escherichia Coli O157:h7, Salmonella Enterica Sp., and Listeria Monocytogenes to Antimicrobial Mixed Micelle Delivery Systems

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SUSCEPTIBILITY OF *ESCHERICHIA COLI* O157:H7, *SALMONELLA ENTERICA* sp., AND *LISTERIA MONOCYTOGENES* TO ANTIMICROBIAL MIXED MICELLE DELIVERY SYSTEMS

A Dissertation Presented

by

CHANELLE N. ADAMS

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DEDICATION

To the greatest examples of love and sacrifice ever witnessed, I dedicate this dissertation to my parents. In as many ways as I can say it, thank you, Gracias, Merci!

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Although the research presented throughout this dissertation is a compilation of experiments performed primarily by myself, I could never have reached this milestone without the support, guidance, and efforts of several people. During my Masters studies, I came across a quote which read "success is 99% failure," and anyone who knows me knows that this has not been an easy journey. Stumbling blocks and feelings of defeat have attempted to impede my progress, however, by the grace of God I made it thru. To my Lord and Savior Jesus Christ, thank you for keeping me in my right mind, and allowing me to reach this point in life. To my parents, Ronald and Cecelia Adams thank you for your constant love and support, the sacrifices you have made for your children have never gone un-noticed, and because of the morals you've instilled complacency and mediocrity have never been an option! Thank you for the pep talks when times got tough and I wanted to quit, and for reminding to "stop all of that crying!" To my favorite sister and brother in the whole wide world, Shalon and Ronald thank you for being there and for keeping me humble when I attempted to boast about how smart I was. I wouldn't trade the two of you for the world. There are two individuals whom I love beyond a shadow of doubt; my niece and nephew Jamia (Mimi) and Robert (Shank)…thank you for being you! The two of you have been the biggest motivating factor throughout this educational journey, and I cannot wait to see the great woman and man you become! A lot of people would not make it in life without grandma's prayers, so to a pair of the strongest women I know, Sarah Ann Blackwell (November 7, 1934 – June 30, 2010) and Louise Adams, thank you for keeping my name on your tongue as you conversed with God. Your prayers have definitely allowed me to be kept. To my Godson, Kiylan thank

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My disclaimer: If you've read this far, and did not see your name please charge it to my head and not my heart. There have been so many hands that have made this moment possible and there is no way I can mention everyone. If you continue reading beyond this page, I hope you find it interesting, and possibly learn something new.

ABSTRACT

SUSCEPTIBILITY OF *ESCHERICHIA COLI* O157:H7, *SALMONELLA ENTERICA* sp., AND *LISTERIA MONOCYTOGENES* TO ANTIMICROBIAL MIXED MICELLE DELIVERY SYSTEMS

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Within the food industry, there is an ever increasing demand to improve the quality of food and ensure safety from pathogenic/spoilage microorganisms. Globalization of the food industry in the 1990's, in conjunction with the worldwide shipment of food created a need for the extension of shelf-life and enhanced maintenance of food quality (12). In the United States alone, the Centers for Disease Control and Prevention report that each year, roughly 1 in 6 Americans (or 48 million people) gets sick, 128,000 are hospitalized, and 3,000 die of foodborne diseases; this correlates to approximately $77.7 billion in economic loss annually (17). Thus the demand for innovative techniques to reduce or eliminate the unintentional presence of microorganisms in food products has increased.

N^{α}-Lauroyl-L-arginine ethyl ester monohydrochloride (lauric arginate (LAE)) is a cationic surfactant possessing antimicrobial ability against the proliferation of several microorganisms including bacteria, fungi, and yeasts (58). Composed of lauric acid, L-arginine, and ethanol, LAE is active over a wide pH range (3-7), and the antimicrobial properties have been reported to be derived from its action on the cytoplasmic

membranes of microorganisms (58). Although LAE is an ideal antimicrobial its usage in the food industry is limited due to its instability. LAE tends to precipitate from solutions at non-acid pH (pH >4.5) as well as in the presence of solutions with high ionic strength (4). Previous research has shown that the tendency for LAE to precipitate in aqueous solutions can be overcome by combining LAE with a non-ionic surfactant (Tween 20) to form mixed micelles (5).

The antimicrobial effectiveness of the cationic surfactant N^{α}-Lauroyl-L-arginine ethyl ester monohydrochloride (lauric arginate (LAE)) applied singly or in combination with the anionic surfactant *Tween-80*, and oil-in-water emulsions were studied to compare inhibition of three foodborne pathogens (*Salmonella* sp., *Escherichia coli* O157:H7, and *Listeria monocytogenes*). The influences of both exposure time and the amount of oil upon the minimum inhibitory concentrations (MIC) were evaluated and are presented here.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

Widespread media coverage of larger outbreaks calls into question the safety of the US food supply. According to the Centers for Disease Control and Prevention (CDC), approximately one of four Americans may experience some form of foodborne illnesses each year (17). Acute gastroenteritis affects 250-350 million people in the United States annually, and an estimated 22%-30% of these cases are thought to be foodborne disease: this results in an annual estimated cost to the US economy to be between $2 billion and $4 billion (68). Viruses, bacteria, parasites, and a variety of chemicals are causes of foodborne-disease outbreak, with the leading causes being of viral and bacterial origin (17). Most vulnerable to foodborne diseases are elderly people, pregnant women, immune-compromised people, and children (50).

Since prehistoric times, chemicals, food additives, and other methods of preservation have been utilized to ensure the safety of foods. In the 1990's the food processing industry became much more global, and the worldwide shipment of food created a demand for the extension of shelf-life and enhanced maintenance of food quality. In more recent years, however, antimicrobials have gained more attention and are now being viewed as a primary mode of intervention/inactivation of pathogenic microorganisms in foods (21).

The purpose of this research is to investigate the antimicrobial efficiency of lauric arginate (a generally regarded as safe (GRAS) antimicrobial) upon three known foodborne pathogens: *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria*

monocytogenes. Studies will include the investigation and determination of the minimum inhibitory concentration when applied singly or in combination with a co-surfactant (Tween-80), the antimicrobial efficiency of lauric arginate in the presence of MCT oil-in-water emulsions, and investigations into the mechanistic targets of lauric arginate. Lastly, lauric arginate will be applied to a model food system to investigate its efficacy in an aqueous product.

CHAPTER II

LITERATURE REVIEW

2.1 Foodborne Illness

Foodborne illness, (often times referred to as "foodborne disease", "foodborne infection", or "food poisoning") is a common yet preventable public health problem (17). According to the Centers for Disease Control and Prevention, each year approximately 48,000,000 cases of foodborne illness will occur; this is the equivalent of one in six Americans acquiring some form of foodborne disease, resulting in an estimated 128,000 hospitalizations and 3,000 deaths (17, 73). The population most at risk for foodborne disease are the elderly, pregnant women, immune-compromise individuals, and children, especially children younger than age 5 (17). For groups of people who are more susceptible to foodborne illness, the effects can be devastating, and potentially deadly. Serious long-term effects associated with several common types of food poisoning include: kidney failure, chronic arthritis, and brain and/or nerve damage.

The spectrum of foodborne disease is ever changing. Over 100 years ago, typhoid fever, tuberculosis, and cholera were common foodborne diseases. Improvements in food safety including pasteurization of milk, safe canning, and disinfection of water supplies have oppressed those diseases. More than 250 different foodborne diseases have been described. The majority of cases are of unknown cause; however bacteria and viruses are the most likely causative agents. Currently, eight known pathogens account for the vast majority of illnesses, hospitalizations, and death. The top pathogens include *Norovirus*, *Salmonella* nontyphoidal, *Clostridium perfringens, Campylobacter spp., Staphylococcus aureus, Toxoplasma gondii, Listeria monocytogenes*, and *E. coli* (STEC) O157:H7 (17).

Regardless of the source of contamination, the microbe or toxin will enter the body through the gastrointestinal tract which is most often the site of initial symptoms. Examples of symptoms include nausea, vomiting, abdominal cramps and diarrhea (50). However, some symptoms can become very serious and progress into life-threatening illness. Table 2.1 presents a summary of the diseases and consequences of ingesting food contaminated by one of the three microorganisms included in this work (73).

With the continual reoccurrence of foodborne illnesses and outbreaks, governmental agencies have developed sources of communication for tracking cases of foodborne illness and their causative agents. Sources for estimation include data from Foodborne Diseases Active Surveillance Network (Food Net), National notifiable Diseases Surveillance System (NNDSS), National Center for Health Statistics (NCHS), and incidences are reported in *Morbidity and Mortality Weekly Report* (MMWR) (18, 19, 50). Advances in food safety has resulted in the development of new packaging and processing techniques such as vacuum sealing, flash chilling or freezing of freshly harvested or processed foods, and food labeling of purchase or use dates. The implementation of such processes aid in the extension of shelf-life and also helps consumers recognize safe periods of consumption.(50)

The area for governmental regulation of Food Safety has recently been broadened with the signing of the Food Safety Modernization Act (FSMA) into law by President Obama on January 4, 2011. FSMA is a law which aims to ensure the safety of the U.S. food supply by shifting the focus from responding to contamination to preventing it (72). Prior to the passing of this law, the FDA had no authority to recall food products; with the exception of infant formula, all recalls were on a voluntary basis by food

manufacturers and distributors. Foods that pose a greater risk to food safety will now undergo more frequent inspections, and imported foods will be held to the same standards as domestics. If an importer refuses to undergo U.S. inspection, the FDA has the right to refuse its entrance into the country. Another benefit of FSMA is science based standards for the safe production and harvesting of fruits and vegetables. It is laws such as this that will continue to make the food supply safer, and reduce the occurrence of microbial contamination.

2.2 Common Foodborne Pathogens

2.2.1 Enterobacteriales

The Enterobacteriales is an order of Gram negative bacteria that are rod shaped and facultatively anaerobic; cell dimensions are typically 0.3-1.0 x 1.0-6.0 µm and can possess peritrichous flagella or be non-motile. The family Enterobacteriaceae has over 40 Genera and representatives include: *Escherichia, Salmonella, Shigella, Citrobacter, Klebsiella, Enterobacter, Erwinia, Serratia, Proteus, and Yersinia*. Several biochemical tests are used to identify bacteria belonging to this group including the degradation of sugar by the Embden-Meyerhof pathway, and formic acid fermentation via mixed acid fermentation or butanediol fermentation (78).

Two representative organisms from this order were utilized for experimental purposes and will be discussed in further detail.

2.2.2 *Escherichia coli* O157:H7

An inhabitant of the colon of humans and other warm-blooded organisms, *Escherichia coli* was first isolated in 1885 by the German bacteriologist, Theodor Escherich (38). Infections caused by *E. coli* include gastroenteritis, urinary tract infections, and diarrheal disease by several mechanisms. Six categories (or strains) of diarrheagenic *E. coli* are recognized (78): enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAggEC), diffusely adhering *E. coli* (DAEC) and enterohemorrhagic *E. coli* (EHEC).

In the US, enterohemorrhagic *E. coli* (EHEC) are the most common group of foodborne pathogenic *E. coli*, with predominately serotype O157:H7 strains, although a variety of other EHEC serotypes including O26, O45, O103, O111, O121 and O145 have been recovered from human patients in the United States (14). Pathogenic *E. coli* are placed into the EHEC group by the presence of Shiga-like toxin genes. Most members of the group also have the ability to cause attaching-effacing lesions which contribute to hemorrhagic colitis with severe abdominal pain and cramps followed by bloody diarrhea (78). The Shiga-like toxin I and II (also called verotoxins 1 and 2) have also been implicated in two extra-intestinal diseases: hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (78).

Shiga toxin-producing *Escherichia coli* (STEC) O157 were first recognized as a human enteric pathogen following two outbreaks of hemorrhagic colitis in the USA in 1982. Examination of culture collections in USA and UK identified only two strains of this serotype isolated between 1973 and 1983 and in Canada six O157 stains were isolated from patients with diarrhea between 1978 and 1982. *E. coli* O157 was therefore a cause of human infection before 1982 (38). The earliest probable case of *E. coli* O157 infection recorded was in 1975, when the organism was isolated from a patient with an episode of gross bloody diarrhea. The evolution of *E. coli* O157:H7 may have begun in an enteropathogenic *E. coli* (EPEC) strain of serotype O55:H7. Serotype O55:H7 is a non-Stx-producing organism associated with cases of infantile diarrhea (38).

The virulence of *E. coli* O157 is due in part to the presence of several well-characterized pathogenic mechanisms such as the production of large amounts of Shiga-like toxin, adhesive factors such as the protein intimin, encoded in the locus of enterocyte

effacement (LEE), and several pO157 plasmid-encoded proteins and other chromosomal-encoded genes (38).

2.2.3 *Salmonella enterica*

In 1885, Daniel E. Salmon and Theobald Smith isolated the first strain of *Salmonella* (35). *Salmonella* is a Gram negative, motile, non-spore forming rod. This organism is commonly associated with the intestinal tract of birds, reptiles amphibians and many mammals (23). Human Salmonellosis has been associated with contaminated foods such as beef products, poultry, eggs, egg products, or water, as well as a variety of processed foods.

There are two types of infection caused by this organism: enteric fever, and gastroenteritis (23). Infection caused by *Salmonella* is known as Salmonellosis (*Salmonella* gastroenteritis) which is caused by over 2,000 *Salmonella* serovars (78). With the exception of *Salmonella typhi*, any of the *Salmonella* are potentially capable of causing Salmonellosis (23). Approximately 45,000 cases of Salmonellosis a year are reported in the United States, but due to under diagnosis, its estimated that is actually may be as many as 2-3 million cases annually.

Salmonellosis is often associated with consumption of contaminated foods. Once the bacteria are in the body, the incubation time is only 8-48 hours. The bacteria invade and multiply the intestinal mucosa where they produce an enterotoxin and cytotoxin that destroy the epithelial cells. Abdominal pain, cramps, diarrhea, nausea, vomiting, and fever are the most prominent symptoms, which usually persist for 2-5 days but can last for weeks. During the acute phase of the disease, as many as 1 billion *Salmonella* can be

found per gram of feces (75). Most adult patients recover, but the loss of fluids can cause problems for children and elderly people. Laboratory diagnosis is by isolation of the bacterium from food or patients' stools. Treatment is with fluid and electrolyte replacement. Prevention depends on good food-processing practices, proper refrigeration, and adequate cooking (78).

2.2.4 *Listeria monocytogenes*

Listeria monocytogenes was first described by Murray *et al.* who named it *Bacterium monocytogenes.* It was renamed *Listerella hepatolytica* by Pirie in 1927, and given its present name by him in 1940 (22). Listeria is a Gram-positive nonsporulating, nonencapulated facultatively anaerobic rod which grows between -0.4 and 50°C. In very young cultures it is found in the bacillary form, later becoming predominantly coccoid (23). The organism possesses peritrichous flagella, which give it a characteristic tumbling motility, occurring in a narrow temperature range. When the organism is grown between 20 and 25°C, flagellin is both produced and assembled at the cell surface, but at 37°C flagellin production is markedly reduced (22). Listeria spp. is isolated from a diversity of environmental sources, including soil, water, effluents, a large variety of foods, and the feces of humans and animals. The natural habitat of these bacteria is thought to be decomposing plant matter, in which they live as saprophytes (76).

The genus *Listeria* contains 6 species: *L.monocytogenes, L. innocua, L. seeligeri, L. welshimeri, L. ivanovii, and L. grayi* (23). *Listeria monocytogenes* is the causative agent of listeriosis, a highly fatal opportunistic foodborne infection. Pregnant women, neonates, the elderly, and debilitated or immunocompromised patients in general are

predominantly affected, although the disease can also develop in normal individuals. Invasive listeriosis is usually severe and includes abortion, sepsis, and meningoencephalitis. In addition to humans, *L.monocytogenes* affects many vertebrate species, including birds (22).

2.3 Food Additives & Antimicrobials

Although concern exists from consumers and consumer based interest groups regarding the safety of consuming additives, food processors have often used additives in order to achieve the extended shelf life. Nutritional, sensory quality, and the safety of foods produced worldwide are all controlled by food additives, and these additives can be divided into six major categories. Preservatives are an identified class of food additives, and there are three types used in foods: antimicrobials, antioxidants, and antibrowning agents (12). Traditionally antimicrobials have been used to extend the shelf-life as well as inhibit spoilage microorganisms. However, in more recent years, there has been a shift in their primary usage. Antimicrobials are now being viewed as a primary mode of intervention/inactivation of pathogenic microorganisms in foods (21).

2.3.1 Antimicrobial delivery systems components.

Antimicrobials can be hydrophobic, amphiphilic, or hydrophilic in nature. When antimicrobials are added to food systems, it is important that the antimicrobial is effective in inhibiting growth, contributes little to no off-flavor, and is stable within the food system. If the antimicrobial cannot be effectively dispersed in a food system due to its physiochemical nature, an antimicrobial delivery system may be used. Delivery systems

can be emulsion, micellular or liposomal based systems, often using surfactants to stabilize and allow effective dispersion within a complex food system.

2.3.2 Surfactants

Surfactants (Surface Active Agents) are one of the most common classes of chemicals, and exhibit a range of unique characteristics. Defined as a chemical used to lower the surface/interfacial tension between two liquids (or a liquid and a solid), these molecules are amphiphilic compounds possessing both a hydrophilic "head" and a hydrophobic "tail" (52). Based on the characteristics of the head group, surfactants can be placed into one of four groups: anionic (the surface-active portion of the molecule bears a negative charge), cationic (the surface-active portion bears a positive charge), zwitterionic (both positive and negative charges may be present in the surface-active portion), and nonionic (the surface-active portion bears no apparent ionic charge)(59).

Within the food industry, surfactants are commonly used to aid in the formation and stabilization of oil-in-water emulsions. One of the primary reasons for their usage is because surfactants diffuse in water and adsorb at interfaces between air and water, or at the interface between oil and water (if water is mixed with oil) (36). The hydrophobic tails will extend into the oil phase, while the hydrophilic head remains in the water phase. By aligning into their desired phase at the surface, the surface properties of water at the water-air or water-oil interface are modified (36).

In the aqueous phase, surfactants can spontaneously form aggregates known as micelles. These highly organized structures are self-assembled in a manner in which the hydrophobic tails form the core of the aggregate, and the hydrophilic heads remain in

contact with the surrounding liquid. The chemical structure of the surfactant head will ultimately determine the shape (36).

2.3.3 Micelles

Surfactants can aggregate spontaneously in solution to form thermodynamically stable structures known as association colloids. These structures can be identified as micelles, bilayers, vesicles, and reverse micelles (52). Identified as one of the most important types of association colloids in many food emulsions, micelles are aggregations of self-assembled surfactant molecules dispersed in a liquid (52). Typically, these structures aggregate in such a way that the hydrophilic "head" region is in contact with the surrounding solvent, while the hydrophobic "tail" region is confined to the micelle core. The physical interactions that hold these structures are relatively weak, and as a result they exhibit flexibility (52).

When surfactant concentrations are low, individual molecules are present in solution and are referred to as monomers. A surfactant can only form micelle when its concentration exceeds some critical level known as the critical micelle concentration (CMC); once the CMC is exceeded, tail groups spontaneously come together to avoid the water, and micelle assembly occurs. Environmental stressors usually have no influence on the structure of surfactants. Their size and shape are well-defined, and as a result achievement of CMC levels will not increase the size or shape, but instead increase the number of micelles (52). Concentrations exceeding the CMC will result in changes in the physiochemical properties of a surfactant solution. Examples include surface tension,

turbidity, and osmotic pressure. The cause of the changes in physiochemical properties are due to differences in properties amongst monomers and micelles (52).

Levels exceeding the CMC can cause surfactants to act as emulsifiers allowing a normally insoluble (in the solvent being used) compound to dissolve by incorporating it into the micelle core. When a second surfactant is introduced into solution the resulting structure is termed a "mixed-micellar system" (24). Mixed micelles can be composed of various compounds and the micelle aggregates serve as transporters. Mixed micelles have the capability to increase the solubility of both soluble and insoluble substances in a given medium that would otherwise be insoluble in the continuous phase (29). This increase in solubility indicates the ability of mixed micelles to improve performance properties when compared to formulations that utilize a single surfactant component. Therefore mixed micellar systems would serve as interesting carrier systems for the delivery of antimicrobial compounds (24).

2.3.4 Emulsions

An emulsion is a mixture of two or more liquids that are otherwise immiscible (non-mixable or unblendable). Emulsion based systems are usually comprised of oil and water, and consists of two phases: the dispersed phase and the continuous phase with the boundary between the two referred to as the "interface" (52). Two immiscible, pure liquids cannot form an emulsion, and in order for the system to become stabilized, an emulsifying agent (usually a surface active agent) must be added (59). Energy input through methods such as shaking, stirring, homogenizing, or exposure to power ultrasound is needed to form an emulsion (30) .

Emulsion stability refers to the ability of an emulsion to resist change in its properties over time (52). Most emulsions are unstable, and unlike micelles do not undergo the process of spontaneous assembly. There are four main categories of emulsion instability: flocculation, creaming, coalescence, and Ostwald ripening. Flocculation occurs when there is an attractive force between the droplets, resulting in the formation of bunches, similar to grapes. Coalescence occurs when droplets bump into each other and combine to form a larger droplet, causing the average droplet size increases over time. Creaming is where the droplets rise to the top of the emulsion under the influence of buoyancy or under the influence of centripetal force induced when a centrifuge is used. Ostwald ripening is a process in which smaller particles in solution dissolve and deposit on larger particles to reach a more thermodynamically stable. Over time, emulsions tend to revert to the stable state of the phases comprising the emulsion. An example of this is seen in the separation of the oil and vinegar components of vinaigrette, an unstable emulsion that will quickly separate unless shaken almost continuously (30). An emulsion can only be referred to as a stable system once the size of the droplets does not change significantly with time. This happens when an appropriate surfactant (emulsifier) is added to increase the kinetic stability.

Recently, there has been increased interest within the food industry in either improving or extending the functional performance of foods using novel structured emulsions. These structured emulsions can be produced using simple processing operations (e.g. mixing, homogenizing, and thermal processing). They are thermodynamically unstable systems that tend to break down over time as a result of several physiochemical mechanisms including gravitational separation, flocculation, coalescence, and Ostwald ripening.

Emulsions can be categorized by diameter of droplets formed during processing: Macroemulsions are thermodynamically unstable and have a diameter range of 0.1-100μm; Nanoemulsions are thermodynamically unstable and have a diameter range of 20-100nm; and Microemulsions are thermodynamically stable and have a diameter range of 5-50nm (51).

Microemulsions are the class of emulsions shown to be thermodynamically stable, and reports have suggested that their structure is harmful to bacterial or microbial cells; they can adversely affect the structure and function of the bacterial membrane. The suggestion is made on the premise that bacteria cannot survive in pure fat/oil alone and that water is required for growth and reproduction. In the formation of microemulsions, the water present is effectively bound to the structure restricting access by microorganisms (1).

2.4 Lauric Arginate

Although several naturally occurring antimicrobials exist, are commercially available and applied in food processing, their efficiency, consumer acceptance, and regulation are not well defined (32). Lauric arginate (LAE) is a cationic preservative (surfactant) that has the ability to inhibit the proliferation of several microorganisms, such as bacteria, fungi, and yeasts (58). Although not a naturally occurring antimicrobial, when ingested by humans the compound is hydrolyzed into natural components: lauric acid, L-arginine, and ethanol. The antimicrobial properties of LAE are believed to be due to its action on the cytoplasmic membranes of microorganisms (56). LAE has been reported to cause a disruption of plasma membrane lipid bilayer, altering the metabolic process (56). The U.S. Food and Drug Administration has granted GRAS (Generally

Recognized As Safe) (74), however its use in industry is limited due to several reasons: "its potency as an antimicrobial may be affected if it interacts with anionic components within the food matrix; (ii) it may bind to anionic biopolymers (mucin) within the mouth, leading to perceived bitterness; and (iii) it tends to precipitate from solution at pH>4.5 and high ionic strength" (5). LAE is functional over a wide pH range (pH 3-7) but there is a tendency for the compound to form large aggregates and sediment. Sedimentation during refrigeration has led to concern of LAE application in cold products such as chilled beverages, dressings, sauces, and desserts (5). One way to overcome the problems associated with instability is to complex the LAE with a co-surfactant, and create mixed micellar systems.

2.4.1 Applications of Lauric Arginate in food and food processing

Many microorganisms play an important role in nature, yet there are several that can cause contamination of food and water resulting in foodborne illness. Some of the foods most commonly associated with foodborne illness include: raw meat and poultry, raw eggs, and unpasteurized milk to name a few (17). Contamination of the aforementioned food products represents a continual challenge for the food industry, and as a result, efforts are underway to find effective treatments that will control the contamination of meat and poultry products.

Contamination of ready to eat (RTE) products by *Listeria monocytogenes* has become a huge concern, and as a result the USDA/FSIS has implemented regulations for meat processors including enforcement of a zero-tolerance rule for the presence of *L.monocytogenes* in RTE meat and poultry products (75). Processors must adhere to one

of three alternatives to control *L.monocytogenes*, two of which require the incorporation of *L.monocytogenes* growth inhibitors to the product formulation (63).

LAE has been granted approval by the USDA/FSIS for application on the surface of RTE meat and poultry products. Allowances of up to 44 ppm when applied as a "sprayed lethality in container" require no labelling, and concentrations up to 200 ppm surface treatment must be labelled. LAE, however, is not currently approved for use in dairy products (69, 77). "Generally-Recognized-As-Safe" (GRAS) compounds, including the organic acids and LAE, have been utilized as dipping solutions or as formulation ingredients in RTE meat products to meet the regulatory requirements of the USDA/FSIS.

A sprayed lethality in container (SLIC) method was used to apply LAE to ham surfaces during packaging (42). The research group to perform these studies was Luchansky *et al* and they found that a 5% solution of LAE caused a $\geq$ 5 log reduction on hams within 24 hours at 4°C. They also found that LAE controlled the outgrowth of *L.monocytogenes* for 60 days when the initial inoculum was 3 log CFU/ham and for 28 days when the initial inoculum was 7 log CFU/ham (42, 44).

Taormina *et* al combined LAE with smoke flavor and applied the treatment to vacuum packed frankfurters. Exposure to LAE at 5,000 ppm caused a 4.11 log reduction within 5 minutes at 4.4°C and a > 5 log reduction by 180 minutes (66). The combination of 5,000 ppm LAE and smoke flavor was lethal; survivors were not detected for any treatments with LAE + smoke flavor except within 5 minutes at 4.4°C for *L.monocytogenes*, which remained at 1.75 log CFU/ml (66). Researchers found that the

efficacy of LAE applied to frankfurters was not significantly affected by inoculation level, inoculation method (dipping vs. spot inoculation), concentration (5,000 and 8,000 ppm), presence of smoke flavor, or treatment volumes (66).

When applied to model food systems, the cationic nature of LAE can potentially lead to a reduction in antimicrobial effectiveness due to the possibility of binding with anionic and hydrophobic food components (9). Both Asker and Bonnaud have demonstrated the likelihood of strong electrostatic binding between LAE and anionic biopolymers (i.e. pectin, alginate, carrageenan, and xanthan) via isothermal titration calorimetry (ITC) (5, 9). Woodcock *et al* reported inhibitory effects of LAE antimicrobial activity through studies comparing chocolate and unflavored milk. (79). The effect of different concentrations of LAE in unflavored and chocolate milk was evaluated over a 21 day period. Immediately after post processing, bacterial counts were <2 log CFU/ml. When treated with 200 mg/L of LAE unflavored milk bacterial levels reached 1.43 log CFU/ml after 21 days of storage. These levels correlate to 5.77 log CFU/ml lower than untreated milk incubated for the same amount of time. In regards to chocolate milk, a system in which stabilizers are added and LAE can potentially bind, initial bacterial counts after post processing were <2 log CFU/ml but increased to nearly 8 log CFU/ml after 21 days of storage. When treated with 200 mg/L of LAE bacterial counts were 0.9 log CFU/ml lower than those in the untreated milk at 21 day post processing (79).

Observations obtained through several studies have indicated that higher concentrations of antimicrobials are required in food systems to inhibit microorganisms than in growth media, and as a result new methodologies are being explored. (15, 34, 46, 61, 62) Here we have introduced a brief synopsis of research that has been performed to

investigate food safety through use of antimicrobials. Researchers have incorporated antimicrobials into formulation, have used dipping solutions, and have applied to packaging. Several studies characterized antimicrobial activities involving combinations of surfactants and essential oils (EOs), usually in the form of nanoemulsions or microemulsions, and others have investigated the development of antimicrobial containing packaging. (46). Advances in these fields are essential for ensuring safety.

Understanding the stability of LAE in a complex food system and the physiochemical interaction of the antimicrobial with bacteria and other food components is critical to expand applications of the cationic antimicrobial to a large variety of food products.

. The addition of antimicrobials to food formulations can be performed directly, or by slow release from packaging materials. Although direct addition to formulation results in immediate reduction of bacterial populations, this may not prevent the recovery of injured cells or the growth of cells that were not immediately destroyed (81) Antimicrobial packaging is a technology that has received attention in recent years, and is utilized to inhibit or retard the proliferation of microorganisms in food, resulting in an extension in product shelf life (6, 16, 65). These specialized films can be designed to deliver a continued and gradual release of antimicrobial agents (including LAE) during the storage and distribution of food packaging (47, 53).

Research by Lopez-de-Dicastillo *et al* has focused on the incorporation of LAE in ethylene-vinyl-alcohol copolymers (EVOH). EVOH is a packing material commonly used to provide anaerobic conditions (due to its strong oxygen barrier). The polymer is

used to protect the active agents during storage and triggers their activity on exposure to humid environments (the food product) (39-41).

2.5 Cellular disruption induced by antimicrobials and detection methods

Emulsion based systems are being increasingly used as delivery systems to encapsulate lipophilic compounds such as antitumor agents, anti-inflammatory agents, vitamins, and antimicrobials (82). Ionic surfactants (such as LAE) have been shown to have strong antimicrobial activity; researchers believe that this is due to the ability of ionic surfactants to incorporate into the lipid membrane of microbial cells and disrupt cellular functions (82). Different approaches have been developed to understand the mode of action of ionic surfactants (including LAE), and some methodologies include potential membrane disturbance, alteration of the efflux pumps, and leakage of cytoplasm constituents or structural changes (58).

Hundreds of compounds expressing antimicrobial activity have been reported, and many detection methods used to investigate their activity are also available (21). This leads to difficulties in developing standardized methods for the evaluation of antimicrobial mechanisms. This further leads to complications in comparing results from different laboratories, determination of antimicrobial effectiveness, establishment of minimal inhibitory concentrations (MIC's), and evaluation of antimicrobial spectrum (21).

One technique which is increasing being used for assessing changes in the cellular morphology and physiology of individual bacterial populations is flow cytometry (49). Other utilizations of this technique include assessment of membrane damage, depolarization, bacterial integrity, and cell viability (58).

The understanding that antimicrobials can cause dramatic functional/structural changes to the cellular envelope leading to cellular lysis/leakage, leads to investigating potassium leakage and proton gradient disturbance as a way of assessing antimicrobial affects (58). Optical density measurements can also be used to monitor the effectiveness of an antimicrobial agent by detecting changes in turbidity however, a major pitfall is turbidity is not a definite indicator. Both live and dead cells can be present in solution, so one way to avoid this is to perform cellular survival studies by plating and incubating after exposure to antimicrobial. Automated platers (e.g. spiral plater), and automated readers (e.g. Scan 500), can be used to reduce any variations that can arise from manual plating and counting. To detect any structural changes amongst cell membranes, microscopy (such as fluorescent microscopy and transmission electron microscopy) can be used.

Rodriguez *et al.* have used methodologies mentioned, and results from this research group have led to the conclusion that LAE causes disturbances in membrane potential, structural changes, and loss of cell viability (55). The most profound finding is that no disruption of cells was detected (58).

2.6 Compositional effects upon antimicrobial activity

Organoleptic properties of food matrices can greatly influence the efficiency of antimicrobials. Disruption in any of these properties can have an effect on diffusion, which can prevent an antimicrobial from being uniformly distributed throughout the product. Natural ingredients in products such as proteins, proteases, lipids salts, and metal ions, all have the potential to interfere with antimicrobial activity by interacting with the antimicrobial directly, or with the target pathogen (32). Antimicrobial application at the

MIC can affect organoleptic properties, and when applied at the dosage necessary to inhibit contamination by spoilage organisms, antimicrobials can affect the organoleptic properties beyond consumer acceptance. Microorganisms possess mechanisms which can result in resistance to environmental factors. Using amounts below the M.I.C. can result is antimicrobial resistance, and injured/stressed cells could recover in the presence of inefficient dosages (21).

2.7 Conclusion

Lauric arginate (LAE) is a cationic surfactant with GRAS status that is of great interest in the food industry, however, its utilization in foods and beverages is limited due to its low solubility and bitter taste. With the understanding that foodborne disease is not restricted to one geographic location, novel ways of incorporating this surfactant is necessary. It is the goal of this work to design and develop antimicrobial delivery systems using a combination of emulsification and micelle formation. We hypothesize that the combination of these laboratory techniques will lead to improvements in the utilization of LAE in targeting microbial pathogens. Successful design will be added to food products to increase shelf life and to prevent microbial spoilage/contamination. The goals of this project will be achieved by investigating the stability of LAE as monomer micelles and in combination with an anionic charged co-surfactant, Tween-80 resulting in the formation of a mixed micellar system. After determination of the minimum inhibitory concentration (M.I.C.), micelles/mixed micelles will be used as delivery vehicles for LAE. These experiments will be performed using a broth based system, and after successful determination of M.I.C. values, the system will be applied to emulsions.

Emulsion technology will be implemented to investigate the influence of oil on the efficacy of LAE.

Table 2.1 Summary of Foodborne Disease caused by pathogens used in this study (57)

Organism	Common Name of Illness	Onset Time After Ingesting	Signs & Symptoms	Duration	Food Sources
Salmonella	Salmonellosis	6-48 hours	Diarrhea, fever, abdominal cramps, vomiting	4-7 days	Eggs, poultry, meat, unpasteurized milk or juice, cheese, contaminated raw fruits and vegetables
Listeria monocytogenes	Listeriosis	9-48 hrs. for gastrointestinal symptoms, 2-6 weeks for invasive disease	Fever, muscle aches, and nausea or diarrhea. Pregnant women may have mild flu-like illness, and infection can lead to premature delivery or stillbirth. The elderly or immunocompromised patients may develop bacteremia or meningitis.	Variable	Unpasteurized milk, soft cheeses made with unpasteurized milk, ready-to-eat deli meats
E. coli O157:H7	Hemorrhagic colitis or E. coli O157:H7 infection	1-8 days	Severe (often bloody) diarrhea, abdominal pain and vomiting. Usually, little or no fever is present. More common in children 4 years or younger. Can lead to kidney failure.	5-10 days	Undercooked beef (especially hamburger), unpasteurized milk and juice, raw fruits and vegetables (e.g. sprouts), and contaminated water

Adapted from Centers for Disease Control and Prevention-Food Safety

References

1. **Al-Adham ISI, E. Khalil, N.D. Al-Hmoud, M. Kierans, P.J. Collier.** 2000. Microemulsions are membrane-active, antimicrobial, self-preserving systems. Journal of Applied Microbiology **89:**32-39.

2. **Asker D, Weiss, J., McClements, D.** 2008. Analysis of the interactions of a cationic surfactant (lauric arginate) with an anionic bioplymer (pectin): isothermal titration calorimetry, light scattering, and microelectrophoresis. Langmuir **25:**116-122.

3. **Aymerich T, Picouet PA, Monofort JM.** 2008. Decontamination technologies for meat products. Meat Sci **78:**114-129.

4. **Bonnaud M, Weiss, J., McClements, D.J.** 2010. Interaction of a food-grade cationic surfactant (lauric arginate) with food-grade biopolymers (pectin, carrageenan, xanhan, alginate, dextran, and chitosan). J Agr Food Chem **58:**9770-9777.

5. **Branen LA, Davidson PM, Salminen S, Thorngate III JH.** 2001. Food Additives, 2nd Ed.

6. **Brooks JT, Sowers EG, Wells JG, Greene KD, Griffin PM, Hoekstra RM, Storockbine NA.** 2005. Non-O157 Shiga Toxin-Producing *Escherichia coli* Infections in the United States,1983-2002. J. Infect Dis **192:**1422-1429.

7. **Burt S.** 2004. Essential oils: their antibacterial properties and potential applications in foods--a review. International journal of Food Microbiology **94:**223-253.

8. **Cagri A, Ustunol Z, Ryser ET.** 2004. Antimicrobial edible films and coatings. Journal of Food Protection **67:**833-848.

9. **CDC.** 2013. MMWR. **62:**283-287.

10. **CDC** 2012, Food Safety. [Online.]

11. **CDC** 2013, National Notifiable Disease Surveillance System. [Online.]

12. **Davidson PM, Sofos JN, Branen AL.** 2005. Antimicrobials in Food, 3rd ed. CRC Press, Boca Raton, FL.

13. **Farber JMaPIP.** 1991. *Listeria monocytogenes,* a Food-Borne Pathogen. Microbiological Reviews **55:**476-511.

14. **Freeman BA.** 1985. Burrows Textbook of Microbiology, 22nd Ed. W.B. Saunders Company, Philidephia.

15. **Gaysinsky S.** 2007. Emulsions and Microemulsions as Antimicrobial Delivery Systems. University of Massachusetts Amherst.

16. **Holmberg KB, Johnson B, Kronberg B, Lindman B.** 2003. Surfactants and polymers in Aqueous Solutions, 2nd ed. Wiley, New York, NY.

17. **Industrial snomechanics L** 2011, [Online.]

18. **Juneja VK, Dwivedi HP, Yan X.** 2012. Novel Natural Food Antimicrobials*. Annual Review of Food Science and Technology **3:**381-403.

19. **Karatzas A, Kets, E., Smid, E., Bennik, M.** 2002. The combined action of carvacrol and high hydrostatic pressure on *Listeria monocytogenes* Scott A. Journal of Applied Microbiology **90:**463-469.

20. **Kass EH.** 1987. A Brief Perspective on the Early History of American Infectious Disease Epidemiology. The Yale Journal of Biology and Medicine **60:**341-348.

21. **Kosswig K.** 2005. Surfactants. *In* Wiley, Ullmann's Encyclopedia of Industrial Chemistry.

22. **Law D.** 2000. The history and evoution of *Escherichia coli* O157 and other Shiga toxin-producing *E. coli*. World Journal of Microbiology & Biotechnology **16:**701-709.

23. **Lopez-de-Dicastillo C, Alonso JM, Catala R, Gavara R, Hernandez-Munoz P.** 2010a. Improving the antioxidant protection of packaged food by incorporating natural flavonoids into ethylene-vinyl-alhohol copolymer (EVOH) films. J Agr Food Chem **58:**10958.

24. **Lopez-de-Dicastillo C, Catala R, Gavara R, Hernandez-Munoz P.** 2011. Food applications of active packaging EVOH films containing cyclodextrins for the preferential scavenging of undesirable compounds. J Food Eng **104:**380-386.

25. **Lopez-de-Dicastillo C, Gallur M, Catala R, Gavara R, Hernandez-Munoz P.** 2010b. Immobilization of beta-cyclodextrin in ethylene-viny alcohol copolymer for active food packaging applications. Jounal of Membrane Science **353:**184-191.

26. **Luchansky JB, A.C.S. Porto, F.M. Wallace, and J.E. Call.** 2002. Recovery of *Listeria monocytogenes* from vacuum-sealed packages of frankfurter: comparison of the U.S. Department of Agriculture (USDA) Food Safety and Inspection Service product composite enrichment method, the USDA Agricultural Research Service (ARS) product composite rinse method, and the USDA-ARS packag rinse method. J. Food Prot. **65:**567.

27. **Luchansky JB, J.E. Call, B. Hristova, L. Rumery, . Yoder, and A. Oser.** 2005. Viability of *Listeria monocytogenes* on commercially prepared hams surface treated with acidic calcium sulfate and lauric arginate and stored at 4°C. *Meat Sci* **71:**92-99.

28. **Ma Q, Davidson, Michael, Zhong Qixin.** 2013. Antimicrobial properties of lauric arginate alone or in combination with essential oils in tryptic soy broth and 2% reduced fat milk. International journal of Food Microbiology **166:**77-84.

29. **Marcos B, Aymerich T, Monofort JM, Garriga M.** 2007. Use of antimicrobial biodegradeable packaging to control *Listeria monocytogenes* during storage of cooked ham. International Journal of Food Microbiology **120:**152-158.

30. **Mason DJ, Gant VA** 2013, Flow Cytometry and Microbiology. [Online.]

31. **McCabe-Sellers BJ, Beattie SE.** 2004. Food safety: emerging trends in foodborne illness surveillance and prevention. Journal of the American Dietetic Association **104:**1708-1717.

32. **McClements DJ.** 2005. Food emulsions, principles, practices, and techniques. CRC Press, Boca Raton, FL.

33. **McClements DJ.** 2007. Critical Review of Technique nd Methodologies for Characterization of Emulsion Stability. Crit Rev Food Sci **47:**611-649.

34. **Muriel-Galet V, Lopez-Carballo G, Gavara R, Hernandez-Munoz P.** 2012. Antimicrobial food packaging film based on the release of LAE from EVOH. International Journal of Food Microbiology **157:**239-244.

35. **Rodriguez E.** 2004. Cellular effects of monohydrochloride of L-arginine, Nalpha-lauroyl ethylester (LAE) exposure on *Salmonella typhimurium* and *Staphylococcus aureus*. Journal of Applied Microbiology **96:**903-912.

36. **Rosen MJ.** 2004. Surfactants and Interfacial Phenomena 3rd ed., p. 444, 3rd ed. John Wiley & Sons, Inc., Hoboken, New Jersey.

37. **Shelef L, Jyothi, E., Bulgarellii, M.** 2006. Growth of enteropathogenic and spoilage bacteria in sage-containing broth and foods. J Food Sci **49:**737-740.

38. **Soni K, Nannapaneni, R., Schilling, M., Jackson, V.** 2010. Bactericidal activity of lauric arginate in milk and Queo Fresco cheese against *Listeria monocytogenes* cold growth. Journal of Dairy Science **93:**4518-4525.

39. **Stopforth JD, Visser, D., Zumbrink, R, Van Dijk, L, and Bontenbal, E.W.** 2010. Control of Literia monocytogenes on Cooked Cured Ham by Formulation with a Lactate-Diacetate Blend and Surface Treatment with Lauric Arginate. Journal of Food Protection **73:**552-555.

40. **Suppakul P, Milts J, Sonneveld K, Bigger SW.** 2003. Active packaging technologies with an emphasis on antimicrobial packaging and its applications. J Food Sci **68:**408-420.

41. **Taormina PJ, Doras, W.J.** 2009. Short-Term Bactericidal Efficacy of Lauric Arginate against *Listeria monocytogenes* Present on the Surface of Frankfurters. *J Food Prot* **72:**1216-1224.

42. **Theinsathid P, Visessanguan W, Kruenate J, Kingcha Y, Keeratipibul S.** 2012. Antimicrobial activity of lauric arginate-coated polylactic acid films against *Listeria monocytogenes* and *Salmonella typhimurium* on cooked sliced ham. J Food Sci **77:**145-152.

43. **US FDA** Oct 25, 2013 2013, Food Safety Modernization Act (FSMA). [Online.]

44. **US FDA** 2013, Foodborne Illness: What You Need to Know. [Online.]

45. **USDA** 2005, Agency Response Letter GRAS Notice No. GRN 000164. [Online.]

46. **USDA FSIS.** 2003. Control of *Listeria monocytogenes* in ready-to-eat meat and poultry products; final rule. *Fed. Regist.* **68:**34208-34252.

47. **Vazquez-Boland JA, Kuhn M, Berche P, Chakraborty T, Dominguez-Bernal G, Goebel W, Gonzalez-Zorn B, Wehland J, Kreft J.** 2001. Listeria pathogenesis and molecular virulence determinants. Clinical Microbiology Reviews **14:**584-640.

48. **WHO.** 2009. Safety evaluation of certain food additives. WHO food additives series **60:**39-84.

49. **Willey JM, Sherwood LM, Woolverton CJ.** 2008. Prescott, Harley, and Klein's Microbiology, 7th ed. McGraw-Hill, New York, NY.

50. **Woodcock N, Hammond, B., Ralyea, R., Boor, K.** 2009. Short communication: N [alpha]-lauroyl-L-arginine ethylester monohydrochloride reduces bacterial growth in pasteruized milk. Journal of Dairy Science **92:**4207-4210.

51. **Zhang Y, Yam K, Chikindas M.** 2004. Effective control of *Listeria monocytogenes* by combination of nisin formulated and slowly released into a broth system. International journal of Food Microbiology**:**15-22.

52. **Ziani K, Chang Y, McLandsborough L, McClements DJ.** 2011. Influence of surfactant charge on antimicrobial efficacy of surfactant-stabilized thyme oil nanoemulsions. J Agric Food Chem **59:**6247-6255.

CHAPTER III

RESEARCH OBJECTIVES

3.1 Investigate the effectiveness of lauric arginate alone and in a mixed micelle system

3.2 Investigate the influence of oil upon the antimicrobial effectiveness of mixed micelles using oil-in-water emulsion systems

3.3 Investigate the mechanism of action of lauric arginate on Gram positive and Gram negative bacterial cells

CHAPTER IV

OIL REDUCES THE EFFICACY OF LAURIC ARGINATE IN OIL-IN-WATER EMULSIONS

4.1 Abstract

The antimicrobial effectiveness of the cationic surfactant N^{α}-Lauroyl-L-arginine ethyl ester monohydrochloride (lauric arginate (LAE)) applied singly or in combination with the anionic surfactant *Tween-80*, and oil-in-water emulsions were studied to compare inhibition of three foodborne pathogens (*Salmonella* sp., *Escherichia coli* O157:H7, and *Listeria monocytogenes*). The influences of both exposure time and the amount of oil upon the minimum inhibitory concentrations (MIC) were evaluated. Within 30 seconds of exposure to LAE, an initial 2-3 log reduction was observed with all bacterial strains tested. Initial studies have yielded MIC results of 100 ppm LAE mixed micelle for *S. enterica* and *E.coli* O157:H7, and 40 ppm for *L.monocytogenes.* When incorporated into emulsion, the MIC increased 2.5-5 fold to 300-500 ppm (Gram negative) and 100-200 ppm (Gram positive) depending on fat concentration. Results suggest that the addition of lauric arginate:Tween-80 mixed micelles to oil-in-water emulsions has an effect on the antimicrobial efficiency; however droplet size does not appear to effect efficacy.

4.2 Introduction

Within the food industry, there is an ever increasing demand to improve the quality of food and ensure safety from pathogenic/spoilage microorganisms. Globalization of the food industry in the 1990's, in conjunction with the worldwide shipment of food created a need for the extension of shelf-life and enhanced maintenance of food quality (12). In the United States alone, the Centers for Disease Control and Prevention report that each

year, roughly 1 in 6 Americans (or 48 million people) gets sick, 128,000 are hospitalized, and 3,000 die of foodborne diseases; this correlates to approximately $77.7 billion in economic loss annually (17). Thus the demand for innovative techniques to reduce or eliminate the unintentional presence of microorganisms in food products has increased.

Many natural and processed foods consist either partly or wholly as emulsions, or have been in an emulsified state at some time during their production (52). Recently, there has been increased interest within the food industry in either improving, or extending the functional performance of foods using emulsion based technology. An emulsion is a system consisting of two immiscible liquids (usually oil and water) with one liquid dispersed as small droplets (52), and these systems are being used as delivery systems to encapsulate lipophilic compounds such as antitumor agents, anti-inflammatory agents, vitamins, and antimicrobials (82).

N^{α}-Lauroyl-L-arginine ethyl ester monohydrochloride (lauric arginate (LAE)) is a cationic surfactant possessing antimicrobial ability against the proliferation of several microorganisms including bacteria, fungi, and yeasts (58). Composed of lauric acid, L-arginine, and ethanol, LAE is active over a wide pH range (3-7), and the antimicrobial properties have been reported to be derived from its action on the cytoplasmic membranes of microorganisms (58). Due to its cationic nature, LAE possesses the potential to interact with anionic food components resulting in a reduction of antimicrobial effectiveness, as well as the production of bitter flavors through interactions with mucins in the mouth. Although LAE is an ideal antimicrobial its usage in the food industry is limited due to its instability. LAE tends to precipitate from solutions at non-acid pH (pH >4.5) as well as in the presence of solutions with high ionic strength (4).

Previous research has shown that the tendency for LAE to precipitate in aqueous solutions can be overcome by combining LAE with a non-ionic surfactant (Tween 20) to form mixed micelles (5).

Micelles are aggregates of surfactant monomers in which the hydrophobic tails are assembled pointing towards the interior (away from solvent), while the hydrophilic heads orient towards the water (52). Formation of mixed micelles can also decrease the electric charge on cationic compounds decreasing their bitterness, and improving the mouth feel (37). For this reason, the creation of antimicrobial delivery systems consisting of cationic LAE, and anionic Tween-80 (Polysorbate 80) were developed to aid in the physical stability of LAE. Tween-80 is a nonionic surfactant derived from polyoxylated sorbitol and oleic acid and is viscous but water soluble (70). Although Tween-80 possesses little antimicrobial activity alone, it has been reported to increase bacterial permeability, and enhance antimicrobial activity (70).

In the studies reported here, we describe the antimicrobial efficacy of LAE alone, LAE:Tween-80 mixed micelles, and LAE:Tween-80 in a MCT oil-in-water emulsion at pH 6.5 upon three foodborne pathogens: *Salmonella enterica*, *Escherichia coli* O157:H7, and *Listeria monocytogenes*. Organisms were tested in broth alone with LAE applied singly or in combination with Tween-80, and in emulsions prepared at six concentrations 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, and 3.0% containing LAE:Tween-80. Cell survival was determined by plate enumeration, and analysis also investigated the influence of droplet size on the efficacy of mixed micelles.

4.3 Materials and methods

4.3.1 Bacterial strains and growth conditions.

Three serovars of *Salmonella* enterica (ATCC strains BAA-708 serovar Enteritidis, BAA-709 serovar Michigan, and BAA 710 serovar Montevideo) were grown in tryptic soy broth (TSB-Difco) (32°C), one strain of Escherichia coli O157:H7 (ATCC 43895) was grown in Luria Bertani broth (LB-Difco) (37°C) or TSB, depending upon the experiment, and three strains of *Listeria monocytogenes* (CU DD6824, CU FSL-N1-304, CU FSL-J1-225) were grown in TSB supplemented with 0.6% yeast extract (TSB-YE-Difco). Stock cultures of all organisms were kept at -80°C in 25% glycerol. Working cultures were streaked on either trypic soy agar (TSA, *Salmonella*), Luria Bertani agar (LBA) plates (*E. coli* O157:H7), or tryptic soy agar with 0.6% yeast extract (*L.monocytogenes*) wrapped in parafilm and stored at 4°C for 4 weeks. For experimental purposes, all organisms were grown overnight and OD_{600} adjusted to 0.1 ($\approx 10^8$ CFU/mL) prior to experimentation.

4.3.2 Determination of minimum inhibitory concentration (MIC) (broth).

Working solutions of the antimicrobial or surfactant combination were prepared by diluting the 1% stock solutions in TSB, LB, or TSB-YE to produce final LAE concentrations of 40-110 ppm. An overnight sample of the bacterial culture was diluted to approximately 10^8 CFU/ml in TSB or LB in phosphate buffered saline (8g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 800 ml of distilled H20. Adjust the pH to 7.4 with HCl. Add H_2O to 1L. Autoclave for 20mins. (Molecular Cloning)) and 1% inoculum transferred to test tubes, produce an initial cell concentration of

approximately 10^6 CFU/ml. After exposure for 30s, 24hr, 48hr, 72hr, and 96hr, 50µl samples were removed from test tubes, diluted in PBS, and plated on appropriate agar medium using AUTOPLATE spiral plater (Advanced Instruments INC., Norwood, MA). The MIC was determined by plate enumeration TSB (*Salmonella*) LB (*E. coli* O157:H7), TSB-YE (*L.monocytogenes*). After incubation at 32°C (*Salmonella*, *L.monocytogenes*) or 37°C (*E. coli* O157:H7) for 48h, colonies were counted using the SCAN 500 (Interscience, France). The MIC was defined as the lowest concentration of antimicrobial agent required to inhibit development of visible growth after 24hr of incubation. The minimum detection limit was 2.0×10^1 CFU/ml.

4.3.3 Drop plate screening method*:*

Samples of *E. coli* O157:H7, *S. enterica* Enteritidis, and *L.monocytogenes* Scott A were inoculated in appropriate media and incubated at 32°C for 24h. The cell density was approximately 10^8 CFU/ml, and 1% of the overnight bacterial culture was added to O/W emulsion ranging from 0.5%-3% inoculated with varying concentrations of LAE:Tween-80. Each agar plate was divided into rows, and six 10µl samples were plated onto appropriate agar. Plates were allowed to dry and incubated at 32°C for 24h.

4.3.4 Determination of minimum inhibitory concentration (MIC) (emulsion).

After 24 hours of growth, bacterial strains were diluted to an $O.D_{600}$ of 0.1. 1% of the adjusted overnight culture was inoculated into TSB or TSB-YE containing LAE:Tween-80 mixed micelles (concentrations ranging from 40-1000ppm) and O/W emulsion at concentrations ranging from 0.5%-3.0%. Test tubes were incubated at 32°C and cell survival measured by plate counts.

4.3.5 Log reduction.

Log reduction was calculated using the following formula: Log No – Log N. (log untreated cells – log treated cells). Experiments were performed in triplicates, and mean values used for calculations.

4.3.6 Chemicals.

Lauric Arginate (LAE) was provided by Vedeqsa Group LAMIRSA (Terrassa, Spain) under the commercial name Mirenat-N. Stock solutions were prepared at 1% (v/v) by dissolving LAE in distilled deionized water (ddH_2O). Tween-80 was purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions of *Tween-80* (1% (v/v)) were prepared in 20mM of phosphate buffer pH 6.5. Mixed micelles were prepared by combining 25ml of 1% LAE with 50ml of 1% *Tween-80*; adjusting pH to 6.5 with HCl and filling to a final volume of 100ml with water. All other chemicals and reagents were of analytical grade supplied by Sigma Chemicals Co. (St. Louis, MO.) or Fisher Scientific (Waltham, MA) All antimicrobial solutions were filter sterilized using a 0.45-µm filter (Nalgene, Rochester, NY.) prior to use.

4.3.7 Micelle composition.

To determine the most stable mixed micelle system, stock solutions of 0.25% (2500 ppm) LAE were combined with varying concentrations of *Tween-80*.

4.3.8 Emulsion preparation.

Coarse emulsion was prepared by blending 10% MCT oil, 0.5% *Tween-80,* and 10mM Phosphate buffer pH 6.5 for 2 minutes. Microfluidizer at 9k PSI and 3 passes was utilized

to achieve droplet size of d=140nm, and two-stage homogenizer at 1K PSI, 2 passes was utilized to achieve droplet sizes of d=320nm. Particle size was measured using dynamic light scattering (DSL).

Statistical analysis Three independent repetitions were performed on each organism at varying fat concentrations, exposure times, and levels of antimicrobial treatments. All variability in data are represented as ± the log standard error of the mean.

The effect of exposure time (t) and antimicrobial concentration (c) was evaluated utilizing data with fat concentrations ranging from 0.5% - 1.0% with a 2 way analysis of variance (PROC GLM, SAS). Further, the interaction between time and concentration was highly significant. Partitioning the interaction shows that the differences among the three concentrations were highly significant at each time point.

4.4 Results

4.4.1 Physical stability studies.

Although active across a wide pH range and inhibitory against a wide spectrum, when applied to solutions whose pH level exceeds 4.5 LAE will precipitate from solution (4). As shown in figure 4.1a, and in agreement with findings reported by *Chang et al.*, the incorporation of LAE to 10mM phosphate buffer (pH 6.5) undisturbed at room temperature for 24 hours results in the formation of large colloidal aggregates (20). Asker *at el* previously found that one way of overcoming the problems associated with precipitation of LAE from solution is to pair LAE with a co-surfactant and create mixed micelles (4). In Figure 4.1b results of varying concentrations of Tween-80 added to a constant amount of LAE (0.25%) are shown. After 24 hours of storage at room

temperature, results reveal that 0.25% LAE in combination with 0.5% (5,000 ppm) *Tween-80* (v/v) creates the most physically stable mixed micelle system; confirmed by no visible sedimentation, creaming, or precipitation.

4.4.2 Determination of MICs.

As shown in table 4.1, susceptibility to antimicrobial treatment was strain dependent. The MIC of LAE and LAE:Tween-80 against *S enterica* and *E. coli O157:H7* were 50 ppm and 100 ppm respectively. When applied to the Gram-positive organism, the MIC decreased by nearly half and was determined to be 18 ppm LAE and 40 ppm LAE: Tween-80. When applied alone, LAE has been shown to have difficulties remaining in solution at elevated pH levels, and in order to investigate the influence of both oil and droplet size on microbial susceptibility via emulsion based systems, mixed micelles were utilized.

The drop plate method of analysis was used to screen a working range of mixed micelle to determine MIC in O/W emulsion. Utilization of this method allowed for testing a wide range of concentrations at once, and narrowing the values for predicted MICs. Drop plate was useful in determining the approximate MIC by identifying colony growth in the opaque emulsions, as shown in Figure 4.2 (dark white circle) and no growth (light circle of dried residue from the emulsion). This method indicated that in the presence of the emulsion, the LAE:Tween80 mixed micelles were less effective than when used in broth alone (Table 4.1), indicating that the presence of fat created binding competition.

Upon determination of the approximate MIC via drop plate, levels of cell survival were identified via plating at 30sec, 24hrs, and 96hrs. in MCT O/W emulsion containing 0.5%, 1.0% or 1.5% fat and droplet sizes of d=140 or d=320nm (Tables 4.2-4.7). Again, the

same trends were observed; samples inoculated with both *E. coli* O157:H7 (Tables 4.2-4.3) or *S. enterica* Enteritidis (Tables 4.4 - 4.5) required higher concentrations for inhibition than *L.monocytogenes* (Tables 4.6 – 4.7). In addition, for all organisms tested, increasing fat concentration resulted in decreased efficacy of the LAE:Tween-80 micelles. Thus, in the presence of O/W emulsions higher concentrations of mixed micelles were required to obtain inhibition. No differences were observed in cell survival between droplet sizes with a diameter of 140 nm (Tables 4.2, 4.4, and 4.6) or diameter of d=320 nm (Tables 4.3 4.5, and, 4.7), thus indicating that the influence of fat was based on the concentration of the fat droplets, rather than an effect of the droplet surface area.

4.4.3 Influence of cell permeabilizer on MIC.

Results presented in Figure 4.3 demonstrate the effects of EDTA added to cells of *E. coli* O157:H7 and *S. enterica* Enteritidis in O/W emulsion. Due to its permeabilizing abilities and disruption in LPS of the outer membrane (OM), the addition of EDTA at 0.1mM or 1.0mM resulted in a reduction in MICs of nearly half. MICs decreased from 100 to 50 when no oil was present, and were reduced by half at 0.5-2%. When tested against *L.monocytogenes* (control) no changes in MIC were observed (data not shown). However, stability of emulsion after the addition of EDTA was compromised (figure 4.4) and could possibly be the result of binding competition.

4.4.4 Statistical significance.

Data presented in Tables 4.2-4.19 are the result of triplicate experiments performed on each organism at varying fat concentrations (0%-1.5%) with a two-way analysis of

variance. Each time point represents the average from three repetitions, and the mean ± standard deviation (n=6). In all tested organisms, and at both droplet sizes, concentration was highly significant (<0.0001).

4.5 Discussion

The results of this study clearly demonstrate the inhibitory ability of lauric arginate applied singly or in combination with Tween-80, and incorporated into O/W emulsion against *L.monocytogenes, E. coli* 0157: H7, and *S. enterica*. All treatment applications resulted in at minimum a 2 log reduction within 30 seconds of exposure, and in agreement with Rodriguez *et al* (58), different effects of LAE were observed in the tested Gram-positive and Gram-negative organisms. All MIC's are listed in table 4.1, and it can be observed that *L.monocytogenes* (18 ppm LAE, 40 ppm LAE:Tween-80) showed susceptibility at nearly half the concentration of *E. coli* and *S enterica* (50 ppm LAE, 100 ppm LAE:Tween-80). The differences in susceptibility support Rodriguez hypothesis that the structure of the bacterial cells resulted in different cellular effects when LAE is applied (58).

The working pH for our trials was 6.5, and LAE is effective at a pH range of 3 to 7. Previous studies have shown that when pH levels exceed 4.5, precipitation can and will occur. The formation of mixed micelles (LAE: Tween-80) could be used to improve LAE's functionality and increase solubility in aqueous solutions. The physical stability studies performed by our group indicate that the creation of mixed micelles at a ratio of 1:2 LAE:Tween-80(v/v) can be used to improve the aggregation stability of LAE in aqueous solutions with elevated pH. This finding is in agreement with Asker et al. who

found that the combination of LAE with Tween-20 reduced the likelihood of sedimentation (3).

Minimum Biocidal Concentration (MBC) is defined as the antimicrobial concentration corresponding to at least a 3 log reduction of viable cells (11), whereas MIC is defined as the lowest concentration of an antimicrobial that prevents growth of a microorganism after a specified incubation period (21). In 2004, Brandt *et al* concluded that a greater than three log reduction was achieved and growth of *L.monocytogenes* was inhibited at the same concentrations (10). Brandt *et al* then concluded that the MIC and MBC were defined at the same concentration (10). Consistent with findings presented by Brandt *et al*, when LAE was applied singly or in combination with Tween-80 nearly a 3 log reduction was observed as well as inhibition after 24 hours of incubation. The concentrations of surfactant utilized in the creation of mixed micelles were one part LAE and two parts Tween-80 which suggests that the amount of LAE required for inhibition when used singly did not change when combined with Tween-80. With this understanding, it can be observed in table 4.1 that complexing LAE with Tween-80 did not cause any changes in MIC values.

The antimicrobial activity of surfactants is directly related to their chemical properties. Several research groups have investigated the inhibitory effects of LAE, and variations in MIC values have been reported. Ma *et al* tested. *L.monocytogenes*, *E. coli* O157:H7, and *S. Enteritidis* and found the MICs to be 11.8ppm for both *L.monocytogenes* and *E. coli* O157:H7, and 23.5ppm for *S.* Enteritidis (45). In studies using the same strains, differences in MIC were observed. Our findings suggest MIC values for *L.monocytogenes* of 18ppm and both *E. coli* O157:H7 and *S.* Enteritidis were inhibited at

concentrations of 40ppm. Although the MIC values obtained during our studies are higher than previous studies using the same organisms, the form of LAE varied. LAE is commercially available in different formulations. It can be purchased as a powder, or as a liquid dissolved in propylene glycol. Depending on the manufacturer process, the purity of LAE will vary. Ma *et al* used Mirenat-TT, Asker *et al* (as well as studies performed here) used Mirenat-N, Brandt *et al* used CytoGuard-LA and Sommers *et al* used CytoGuard-STAT-N. Mirenat-TT contains 15 $\pm$ 0.5% w/w LAE, Mirenat-N contains 10.5% w/v LAE in propylene glycol solvent, and CytoGuard-LA contains 10% w/w LAE, along with a mixture of growth inhibitors made to improve the effectiveness of LAE. Some of the differences that could contribute to observable variations are: 1) Stock solutions of Mirenat-TT were prepared in 70% ethanol, 2) stock solutions of Mirenat-N were prepared in water, and 3) MIC values were determined by OD measurements.

Results presented by several researchers indicate the effectiveness of LAE against tested pathogens, however the combination of LAE with other antimicrobials varied. Some used essential oils, liquid smoke, or surfactants. Depending on the antimicrobial effectiveness of the combined ingredients, the efficacy of LAE could be enhanced. Different approaches in MIC determination, variations in the forms of LAE utilized, and combinations of LAE with various chemicals would possibly explain variations in MIC values observed.

Understanding that the bacterial cell's susceptibility to LAE is dependent upon the bacterial cell wall, we investigated the use of a known cell wall permeabilizer (EDTA) to destabilize the outer membrane (OM) of *E. coli* 0157:H7 and *S. enterica*. Studies performed by Alakomi (2, 3) and Helander (26) utilizing *Pseudomonas, Salmonella*, and

E. coli have shown that the addition of EDTA to gram-negative organisms causes release of lipopolysaccharide (LPS) and disintegration of the OM structure. The incorporation of permeabilizers in biocide formulations enabled the use of decreased concentrations of the active biocide ingredients. The effect of EDTA on the OM properties of *E. coli* O 157: H7 and *S enterica* Enteritidis was applied to our O/W emulsion system and reductions in MIC of mixed micelle was observed. As a control *L.monocytogenes* was tested and no changes in MIC were observed (data not shown).

In the presence of EDTA, both *E. coli* O157:H7 and *Salmonella sp.* became more susceptible to LAE:Tween-80 inhibition (Figure 4-3). MIC values were reduced by half for fat concentrations ranging from 0.5 to 2.0%. EDTA is a chelator and would bind divalent cations present, thus increasing the exposure of negatively charged regions, potentially allowing positively charged LAE to work more efficiently. However, issues with emulsion stability were heightened when EDTA was added at these fat concentrations (figure 4-4). This instability was observed only when mixed micelle was added leaving us to believe there is some competition for binding occurring. Although instability was observed, inhibition was maintained.

The antimicrobial activity of LAE against a wide spectrum of microorganisms has made it an ideal ingredient within the food industry. The results presented throughout this paper suggest that the addition of LAE in combination with Tween-80 to aqueous based systems can aid in the protection of microbial contamination by *E. coli* 0157: H7, *S. enterica*, and *L.monocytogenes*, and the effectiveness of LAE against Gram negative bacteria can be enhanced by the addition of a chelating agent, such as EDTA. Future

studies should investigate the stability of these mixed micelle systems during long term storage.

Table 4.1 [1]Bacterial strains were inoculated with varying amounts of LAE alone, or LAE:Tween-80 (mixed micelle), incubated at 32°C or 37°C, and samples taken every 24 hours for 96 hours. Cell survival was determined by plate enumeration.

Bacterium	Isolate	LAE MIC[1] (ppm)	LAE:Tween-80 MIC[1] (ppm)
Gram negative			
Salmonella enterica serovar Enteritidis	ATCC BAA-708	50	100
Salmonella enterica serovar Michigan	ATCC BAA-709	50	100
Salmonella enterica serovar Montevideo	ATCC BAA-710	50	100
Escherichia coli O157:H7	ATCC 43895	50	100
Gram positive			
Listeria monocytogenes (LM 9)	CU DD6824	18	40
Listeria monocytogenes (LM 10)	CU FSL-N1-304	16	40
Listeria monocytogenes (LM 21-Scott A)	CU FSL-J1-225	18	40

Table 4.2 Antimicrobial effectiveness of LAE:Tween-80 mixed micelles against *E. coli* O157:H7 in media containing emulsion at varying concentrations and droplet size of d=140nm at 32°C for 30s, 24 and 96 hrs. Values expressed as logarithm of colony forming units (log CFU/ml) and log standard error of the mean

Fat concentration (d=140nm)	LAE:Tween-80 concentration (ppm)	Exposure time	Cell Survival (Log CFU/ml)	Log Standard Error of the Mean (SEM)	Log Reduction (Log N_0 – Log N)
0%	100 ppm	30sec.	6.95	±0.02	0.51
		24 hrs.	1.30	0	7.18
		96 hrs.	1.30	0	7.30
0.5%	0 ppm-control	30 sec.	7.23	±0.06	0
		24 hrs.	8.88	±0.01	0
		96 hrs.	8.34	±0.54	0
	100 ppm	30 sec.	3.14	±0.07	4.10
		24 hrs.	4.40	±0.11	4.48
		96 hrs.	5.51	±0.10	2.83
	250 ppm	30 sec.	2.98	±0.85	4.26
		24 hrs.	1.30	0	7.58
		96 hrs.	1.30	0	7.04
1.0%	0 ppm-control	30 sec.	7.26	±0.04	0
		24 hrs.	8.86	±0.05	0
		96 hrs.	8.14	±0.36	0
	100 ppm	30 sec.	6.29	±0.08	0.97
		24 hrs.	9.70	±0.06	0
		96 hrs.	8.42	±0.08	0
	400 ppm	30 sec.	2.21	±0.91	5.05
		24 hrs.	1.30	0	7.56
		96 hrs.	1.30	0	6.84
1.5%	0 ppm-control	30 sec.	7.24	±0.03	0
		24 hrs.	8.88	±0.04	0
		96 hrs.	8.74	±0.11	0
	100 ppm	30 sec.	6.23	±0.06	1.01
		24 hrs.	9.79	±0.09	0
		96 hrs.	8.70	±0.09	0.49
	500 ppm	30 sec.	2.13	±0.83	5.11
		24 hrs.	1.30	0	7.58
		96 hrs.	1.30	0	7.44

Two-way ANOVA was used to measure the effects of time, and concentration: bacteria susceptibility in 0/W emulsion. Each time point represents the average from three repetitions, and the mean ± standard deviation (n=6). Each gated area denotes a specific dataset analyzed independently of one another. Statistical significance can be found in Table 4.8.

Table 4.3 Antimicrobial effectiveness of LAE:Tween-80 mixed micelles against *E. coli* O157:H7 in media containing emulsion at varying concentrations and droplet size of d=320nm at 32°C for 30s, 24 and 96 hrs. Values expressed as logarithm of colony forming units (log CFU/ml) and log standard error of the mean

Fat concentration (d=320nm)	LAE:Tween-80 concentration (ppm)	Exposure time	Cell Survival (Log CFU/ml)	Log Standard Error of the Mean (SEM)	Log Reduction (Log N_0 – Log N)
0%	100 ppm	30sec.	6.95	±0.02	0.51
		24 hrs.	1.30	0	7.18
		96 hrs.	1.30	0	7.30
0.5%	0 ppm-control	30 sec.	7.24	±0.06	0
		24 hrs.	8.88	±0.01	0
		96 hrs.	8.34	±0.54	0
	100 ppm	30 sec.	6.25	±0.05	0.99
		24 hrs.	8.20	±0.61	0.67
		96 hrs.	8.49	±0.06	0
	250 ppm	30 sec.	5.97	±0.06	1.27
		24 hrs.	1.30	0	7.58
		96 hrs.	1.30	0	7.04
1.0%	0 ppm-control	30 sec.	7.26	±0.04	0
		24 hrs.	8.86	±0.05	0
		96 hrs.	8.14	±0.36	0
	100 ppm	30 sec.	6.29	±0.08	0.97
		24 hrs.	9.70	±0.06	0
		96 hrs.	8.42	±0.08	0
	400 ppm	30 sec.	5.94	±0.11	1.32
		24 hrs.	1.30	0	7.56
		96 hrs.	1.30	0	6.84
1.5%	0 ppm-control	30 sec.	7.24	±0.03	0
		24 hrs.	8.88	±0.04	0
		96 hrs.	8.74	±0.11	0
	100 ppm	30 sec.	6.23	±0.06	1.01
		24 hrs.	9.79	±0.09	0
		96 hrs.	8.70	±0.09	0.04
	500 ppm	30 sec.	6.07	±0.06	1.17
		24 hrs.	1.30	0	7.58
		96 hrs.	1.30	0	7.44

Two-way ANOVA was used to measure the effects of time, and concentration: bacteria susceptibility in 0/W emulsion. Each time point represents the average from three repetitions, and the mean ± standard deviation (n=6). Each gated area denotes a specific dataset analyzed independently of one another. Statistical significance can be found in Table 4.8.

Table 4.4 Antimicrobial effectiveness of LAE:Tween-80 mixed micelles against *S. enterica* Enteritidis in media containing emulsion at varying concentrations and droplet size of d=140nm at 32°C for 30s, 24 and 96 hrs. Values expressed as logarithm of colony forming units (log CFU/ml) and log standard error of the mean.

Fat concentration (d=140nm)	LAE:Tween-80 concentration (ppm)	Exposure time	Cell Survival (Log CFU/ml)	Log Standard Error of the Mean (SEM)	Log Reduction (Log N_0 – Log N)
0%	100 ppm	30 sec.	6.70	±0.12	1.86
		24 hrs.	1.30	0	8.13
		96 hrs.	1.30	0	8.2
0.5%	0 ppm- control	30 sec.	8.43	±0.25	0
		24 hrs.	9.39	±0.15	0
		96 hrs.	9.44	±0.27	0
	100 ppm	30 sec.	2.26	±0.71	6.17
		24 hrs.	9.45	±0.36	0
		96 hrs.	9.31	±0.85	0.13
	250 ppm	30 sec.	1.64	±0.11	6.78
		24 hrs.	1.30	0	8.08
		96 hrs.	1.30	0	8.14
1.0%	0 ppm-control	30 sec.	8.43	±0.25	0
		24 hrs.	9.39	±0.15	0
		96 hrs.	9.44	±0.27	0
	100 ppm	30 sec.	2.29	±0.46	6.14
		24 hrs.	9.48	±0.07	0
		96 hrs.	9.99	±0.21	0
	400 ppm	30 sec.	1.64	±0.26	6.78
		24 hrs.	1.30	0	8.08
		96 hrs.	1.30	0	8.14
1.5%	0 ppm-control	30 sec.	8.43	±0.25	0
		24 hrs.	9.39	±0.15	0
		96 hrs.	9.44	±0.27	0
	100 ppm	30 sec.	2.12	±0.58	6.31
		24 hrs.	9.30	±0.04	0.09
		96 hrs.	9.99	±0.02	0
	400 ppm	30 sec.	3.64	±0.04	4.79
		24 hrs.	1.65	±0.35	7.73
		96 hrs.	1.30	0	8.14

Two-way ANOVA was used to measure the effects of time, and concentration: bacteria susceptibility in 0/W emulsion. Each time point represents the average from three repetitions, and the mean ± standard deviation (n=6). Each gated area denotes a specific dataset analyzed independently of one another. Statistical significance can be found in Table 4.8.

Table 4.5 Antimicrobial effectiveness of LAE:Tween-80 mixed micelles against *S. enterica* Enteritidis in media containing emulsion at varying concentrations and droplet size of d=320nm at 32°C for 30s, 24 and 96 hrs. Values expressed as logarithm of colony forming units (log CFU/ml) and log standard error of the mean.

Fat concentration (d=320nm)	LAE:Tween-80 concentration (ppm)	Exposure time	Cell Survival (Log CFU/ml)	Log Standard Error of the Mean (SEM)	Log Reduction (Log N_0 – Log N)
0%	100 ppm	30 sec.	6.70	±0.12	1.86
		24 hrs.	1.30	0	8.13
		96 hrs.	1.30	0	8.2
0.5%	0 ppm-control	30 sec.	8.43	±0.25	0
		24 hrs.	9.39	±0.15	0
		96 hrs.	9.44	±0.27	0
	100 ppm	30 sec.	6.11	±0.03	2.31
		24 hrs.	10.61	±0.31	0
		96 hrs.	8.13	±0.61	1.31
	250 ppm	30 sec.	5.20	±0.15	3.22
		24 hrs.	1.30	0	8.08
		96 hrs.	1.30	0	8.14
1.0%	0 ppm-control	30 sec.	8.43	±0.25	0
		24 hrs.	9.39	±0.15	0
		96 hrs.	9.44	±0.27	0
	100 ppm	30 sec.	6.23	±0.04	2.20
		24 hrs.	10.73	±0.03	0
		96 hrs.	8.75	±0.13	0.69
	400 ppm	30 sec.	1.64	±0.26	6.78
		24 hrs.	1.30	0	8.08
		96 hrs.	1.30	0	8.14
1.5%	0 ppm-control	30 sec.	8.43	±0.25	0
		24 hrs.	9.39	±0.15	0
		96 hrs.	9.44	±0.27	0
	100 ppm	30 sec.	6.30	±0.03	2.13
		24 hrs.	10.80	±0.11	0
		96 hrs.	7.59	±0.61	1.85
	500 ppm	30 sec.	4.65	±0.35	3.77
		24 hrs.	1.30	0	8.08
		96 hrs.	1.30	0	8.14

Two-way ANOVA was used to measure the effects of time, and concentration: bacteria susceptibility in 0/W emulsion. Each time point represents the average from three repetitions, and the mean ± standard deviation (n=6). Each gated area denotes a specific dataset analyzed independently of one another. Statistical significance can be found in Table 4.8.

Table 4.6 Antimicrobial effectiveness of LAE:Tween-80 mixed micelles against *L.monocytogenes* Scott A in media containing emulsion at varying concentrations and droplet size of d=140nm at 32°C for 30s, 24 and 96 hrs. Values expressed as logarithm of colony forming units (log CFU/ml) and log standard error of the mean.

Fat concentration (d=140nm)	LAE:Tween-80 concentration (ppm)	Exposure time	Cell Survival (Log CFU/ml)	Log Standard Error of the Mean (SEM)	Log Reduction (Log N_0 – Log N)
0%	40 ppm	30 sec.	5.95	±0.04	2.01
		24 hrs.	1.30	0	7.3
		96 hrs.	1.30	0	6.97
0.5%	0 ppm-control	30 sec.	7.96	±0.48	0
		24 hrs.	8.60	±0.17	0
		96 hrs.	8.27	±0.09	0
	40 ppm	30 sec.	6.14	±0.02	1.82
		24 hrs.	8.51	±0.02	0.08
		96 hrs.	8.26	0	0.01
	100 ppm	30 sec.	5.91	±0.07	2.05
		24 hrs.	1.30	0	7.29
		96 hrs.	1.30	0	6.96
1.0%	0 ppm-control	30 sec.	7.96	±0.48	0
		24 hrs.	8.60	±0.17	0
		96 hrs.	8.27	±0.09	0
	40 ppm	30 sec.	6.26	±0.05	1.70
		24 hrs.	8.28	±0.11	0.31
		96 hrs.	8.28	0	0
	200 ppm	30 sec.	2.87	±0.21	5.09
		24 hrs.	1.30	0	7.29
		96 hrs.	1.30	0	6.96
1.5%	0 ppm-control	30 sec.	7.96	±0.48	0
		24 hrs.	8.60	±0.17	0
		96 hrs.	8.27	±0.09	0
	40 ppm	30 sec.	6.18	±0.05	1.78
		24 hrs.	8.24	±0.06	0.35
		96 hrs.	8.37	±0.03	0
	200 ppm	30 sec.	2.74	±0.10	5.22
		24 hrs.	1.30	0	7.29
		96 hrs.	1.30	0	6.96

Two-way ANOVA was used to measure the effects of time, and concentration: bacteria susceptibility in 0/W emulsion. Each time point represents the average from three repetitions, and the mean ± standard deviation (n=6). Each gated area denotes a specific dataset analyzed independently of one another. Statistical significance can be found in Table 4.8.

Table 4.7 Antimicrobial effectiveness of LAE:Tween-80 mixed micelles against *L.monocytogenes* Scott A in media containing emulsion at varying concentrations and droplet size of d=320nm at 32°C for 30s, 24 and 96 hrs. Values expressed as logarithm of colony forming units (log CFU/ml) and log standard error of the mean.

Fat concentration (d=320nm)	LAE:Tween-80 concentration (ppm)	Exposure time	Cell Survival (Log CFU/ml)	Standard Error of the Mean (SEM)	Log Reduction (Log N_0 – Log N)
0%	40 ppm	30 sec.	5.95	±0.04	2.01
		24 hrs.	1.30	0	7.3
		96 hrs.	1.30	0	6.97
0.5%	0 ppm-control	30 sec.	7.96	±0.48	0
		24 hrs.	8.60	±0.17	0
		96 hrs.	8.27	±0.09	0
	40 ppm	30 sec.	6.33	±0.05	1.63
		24 hrs.	9.56	±0.12	0
		96 hrs.	8.40	±0.02	0
	100 ppm	30 sec.	6.05	±0.09	1.91
		24 hrs.	1.30	0	7.29
		96 hrs.	1.30	0	6.96
1.0%	0 ppm-control	30 sec.	7.96	±0.48	0
		24 hrs.	8.60	±0.17	0
		96 hrs.	8.27	±0.09	0
	40 ppm	30 sec.	6.21	±0.11	1.75
		24 hrs.	9.58	±0.05	0
		96 hrs.	8.46	±0.04	0
	200 ppm	30 sec.	5.78	±0.11	2.18
		24 hrs.	1.30	0	7.29
		96 hrs.	1.30	0	6.96
1.5%	0 ppm-control	30 sec.	7.96	±0.48	0
		24 hrs.	8.60	±0.17	0
		96 hrs.	8.27	±0.09	0
	40 ppm	30 sec.	6.10	±0.01	1.86
		24 hrs.	9.54	±0.06	0
		96 hrs.	8.17	±0.12	0.10
	200 ppm	30 sec.	6.06	±0.23	1.90
		24 hrs.	1.30	0	7.30
		96 hrs.	1.30	0	6.96

Two-way ANOVA was used to measure the effects of time, and concentration: bacteria susceptibility in 0/W emulsion. Each time point represents the average from three repetitions, and the mean ± standard deviation (n=6). Each gated area denotes a specific dataset analyzed independently of one another. Statistical significance can be found in Table 4.8.

Table.4.8 Statistical analysis of *E. coli* O157:H7 grown in the presence of LAE:Tween-80 O/W emulsion with varying fat concentrations and droplet sizes

E. coli 140nm 0.5%

T	p>0.05	ns
C	P<0.0001	s
T*C	P<0.0001	s

E. coli 140nm 1.0%

T	P<0.001	s
C	P<0.0001	s
T*C	P<0.0001	s

E. coli 320nm 0.5%

T	p>0.05	ns
C	P<0.0001	s
T*C	P<0.0001	s

E. coli 320nm 1.0%

T	P<0.0001	s
C	P<0.0001	s
T*C	P<0.0001	s

E. coli 140nm 1.5%

T	P<0.001	s
C	P<0.0001	s
T*C	P<0.0001	s

E. coli 320nm 1.5%

T	P<0.0001	s
C	P<0.0001	s
T*C	P<0.0001	s

The effects of time (T) and concentration (C) after exposure to LAE: Tween-80 in O/W emulsions was analyzed with a two-way ANOVA (PROC GLM, SAS). P value <0.05 was considered to be statistically significant (ns = not significant s = significant).

Table.4.9 Statistical analysis of *L.monocytogenes* Scott A grown in the presence of LAE:Tween-80 O/W emulsion with varying fat concentrations and droplet sizes.

LM 21 140nm 0.5%

T	P>0.05	ns
C	P<0.0001	s
T*C	P>0.05	ns

LM 21 140nm 1.0%

T	P>0.05	ns
C	P<0.0001	s
T*C	P>0.05	ns

LM 21 320nm 0.5%

T	P<0.0001	s
C	P<0.0001	s
T*C	P<0.0001	s

LM 21 320nm 1.0%

T	P<0.0001	s
C	P<0.0001	s
T*C	P<0.0001	s

LM 21 140nm 1.5%

T	P>0.05	ns
C	P<0.0001	s
T*C	P>0.05	ns

LM 21 320nm 1.5%

T	P<0.0001	s
C	P<0.0001	s
T*C	P<0.0001	s

The effects of time (T) and concentration (C) after exposure to LAE: Tween-80 in O/W emulsions was analyzed with a two-way ANOVA (PROC GLM, SAS). P value <0.05 was considered to be statistically significant (ns = not significant s = significant).

Table.4.10 Statistical analysis of *S. enterica* Enteritidis grown in the presence of LAE:Tween-80 O/W emulsion with varying fat concentrations and droplet sizes.

S. enterica 140nm 0.5%

T	P<0.0001	s
C	P<0.0001	s
T*C	P<0.0001	s

S. enterica 320nm 0.5%

T	P<0.001	s
C	P<0.0001	s
T*C	P<0.0001	s

S. enterica 140nm 1.0%

T	P<0.0001	s
C	P<0.0001	s
T*C	P<0.0001	s

S. enterica 320nm 1.0%

T	P<0.01	s
C	P<0.0001	s
T*C	P<0.0001	s

S. enterica 140nm 1.5%

T	P<0.0001	s
C	P<0.0001	s
T*C	P<0.0001	s

S. enterica 320nm 1.5%

T	P<0.001	s
C	P<0.0001	s
T*C	P<0.0001	s

The effects of time (T) and concentration (C) after exposure to LAE: Tween-80 in O/W emulsions was analyzed with a two-way ANOVA (PROC GLM, SAS). P value <0.05 was considered to be statistically significant (ns = not significant s = significant).

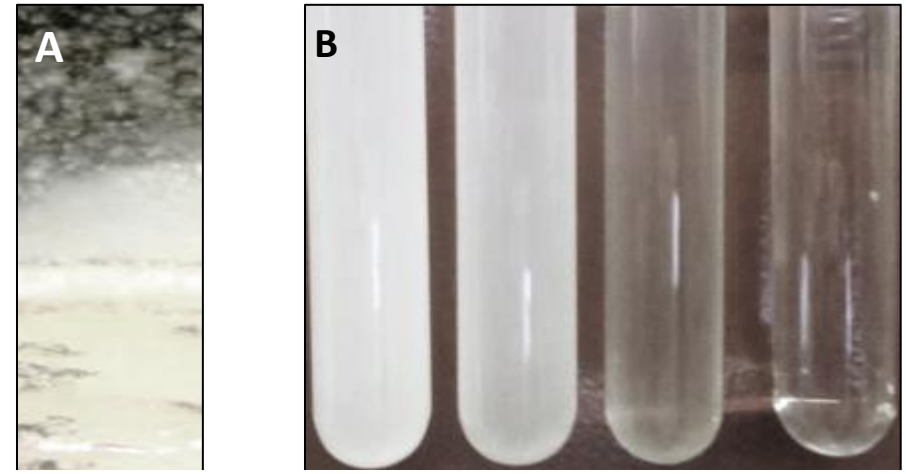


Figure 4.1. Visual appearance of aqueous solutions containing a)LAE alone and b) LAE:*Tween-80* complexes (pH 6.5) with 0.25% LAE and varying ratios of Tween-80 as indicated

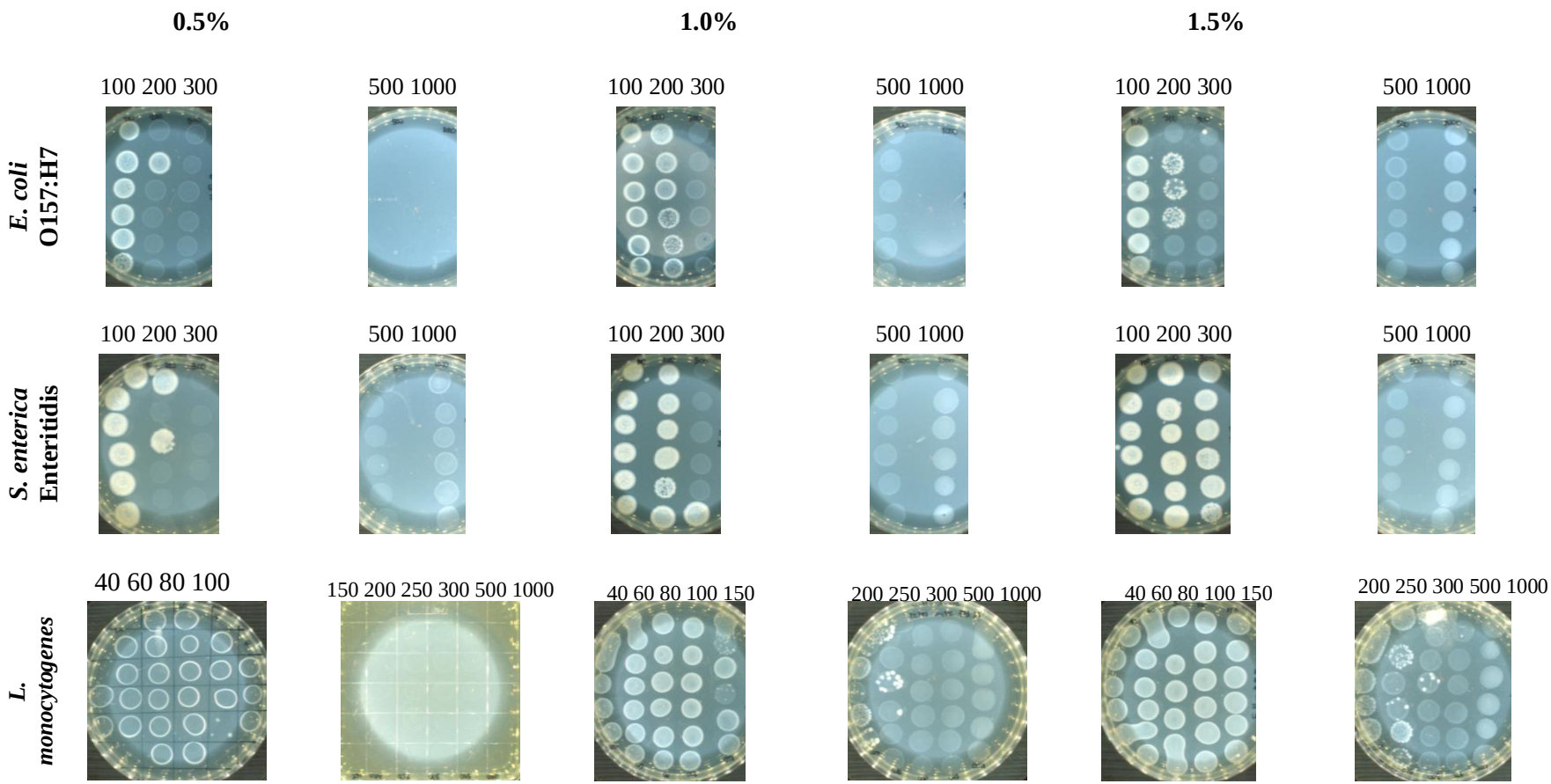


Figure 4.2: Screening experiment to determine approximate MIC in opaque emulsions. Drop plate samples (10 µl) of undiluted *Salmonella enterica* Enteritidis, *E. coli* O157:H7, and *L.monocytogenes* Scott A in O/W emulsions after a 24h exposure to concentrations of LAE:Tween-80. Dark drops represent bacterial growth, light drops are indicative of dried emulsion without bacterial growth. Range of MIC was then confirmed using standard plating experiments.

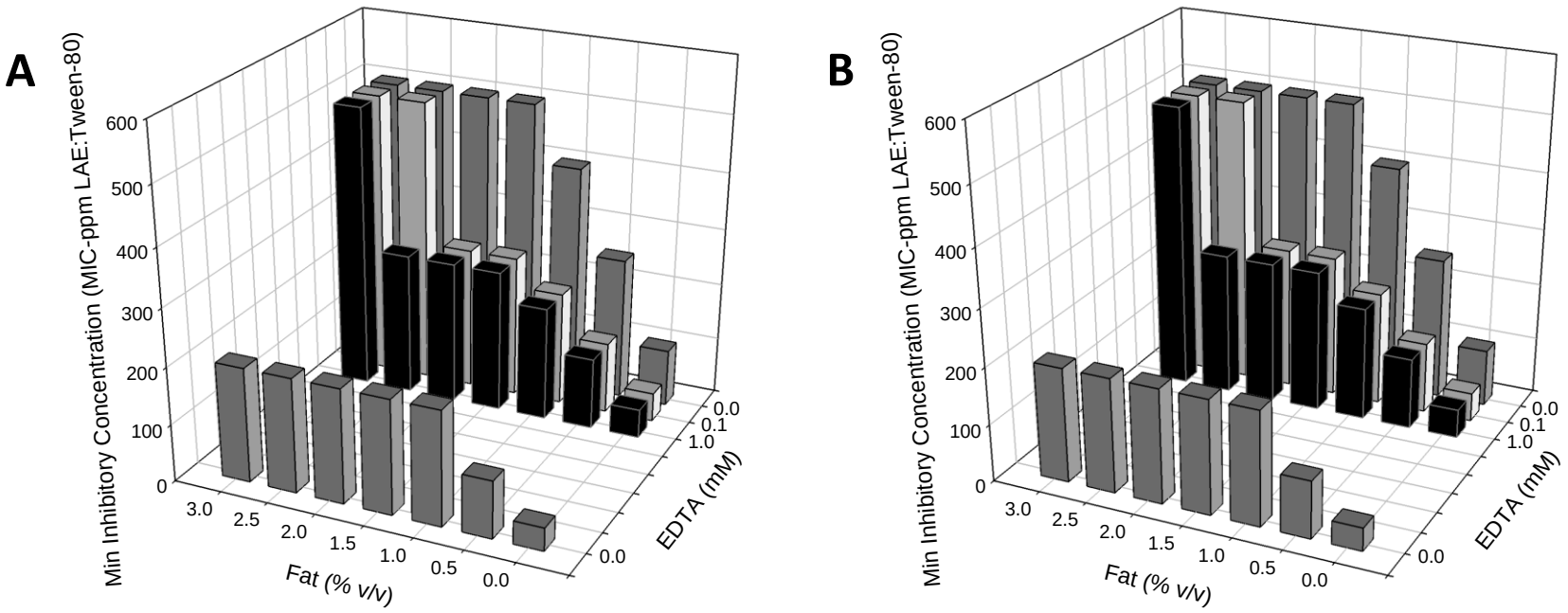
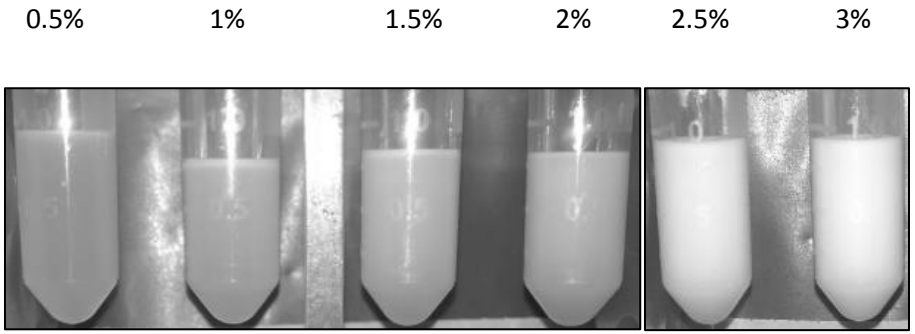


Figure 4.3 Influence of fat at varying concentrations on the survival in the presence and absence of EDTA. A) *L.monocytogenes (single dark gray column, 0 EDTA)* and *E. coli* O157:H7 (*three EDTA concentrations*), and (B) *Listeria monocytogenes (single gray column, 0 EDTA) S. enterica* Enteritidis (*three EDTA concentrations*).



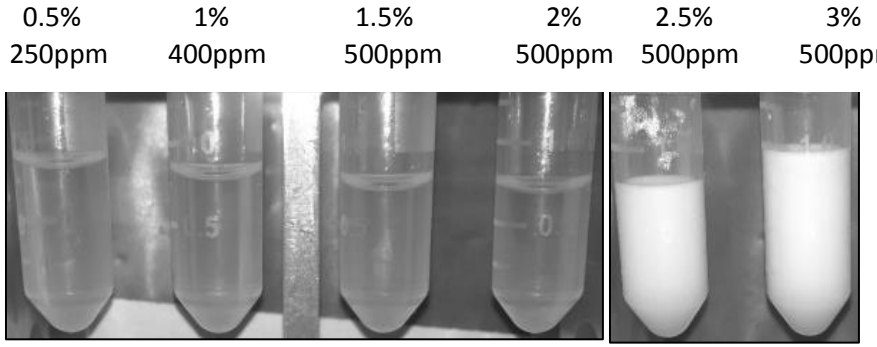


Figure 4.4a: Screening experiment to determine influence of 0.1mM EDTA on *E. coli* O157:H7 susceptibility to LAE:Tween-80 in O/W emulsion. After a 24h exposure to varying concentrations of fat only (top row) and fat + LAE:Tween-80 (bottom row). The addition of EDTA to the O/W emulsion resulted in emulsion breakdown.

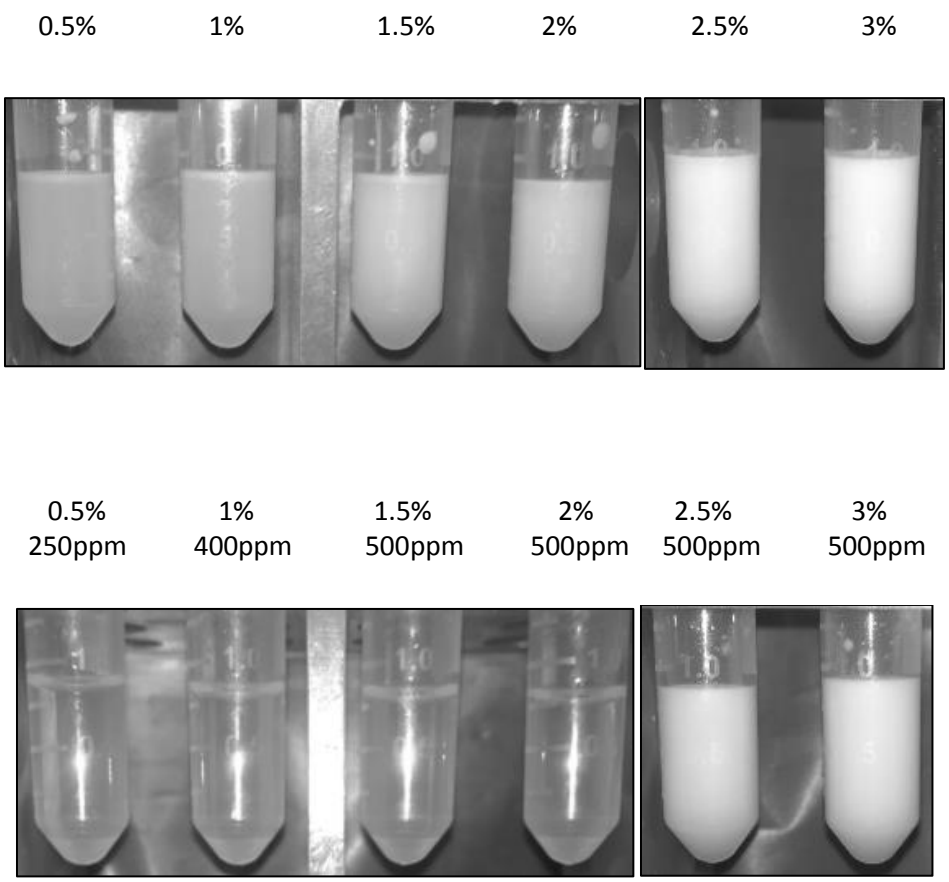


Figure 4.4b: Screening experiment to determine influence of 1.0mM EDTA on *E. coli* O157:H7 susceptibility to LAE:Tween-80 in O/W emulsion. After a 24h exposure to varying concentrations of fat only (top row) and fat + LAE:Tween-80 (bottom row). The addition of EDTA to the O/W emulsion resulted in emulsion breakdown.

References

1. **Alakomi HL.** 2003. Effect of EDTA on *Salmonella enterica* serovar Typhimurium involves a component not assignable to lipopolysaccharide release. Microbiology **149:**2015-2021.

2. **Alakomi HL, Paananen A, Suihko ML, Helander IM, Saarela M.** 2006. Weakening effect of cell permeabilizers on Gram-negative bacteria causing biodeterioration. Appl Environ Microbiol **72:**4695-4703.

3. **Asker D, Weiss J, McClements DJ.** 2011. Formation and stabilization of antimicrobial delivery systems based on electrostatic complexes of cationic-non-ionic mixed micelles and anionic polysaccharides. J Agric Food Chem **59:**1041-1049.

4. **Asker D, Weiss, J., McClements, D.** 2008. Analysis of the interactions of a cationic surfactant (lauric arginate) with an anionic bioplymer (pectin): isothermal titration calorimetry, light scattering, and microelectrophoresis. Langmuir **25:**116-122.

5. **Brandt AL, Castillo A, Harris KB, Keeton JT, Hardin MD, Taylor TM.** 2010. Inhibition of Listeria monocytogenes by food antimicrobials applied singly and in combination. J Food Sci **75:**M557-563.

6. **Branen JK, Davidson PM.** 2004. Enhancement of nisin, lysozyme, and monolaurin antimicrobial activities by ethylenediaminetetraacetic acid and lactoferrin. International Journal of Food Microbiology **90:**63-74.

7. **Branen LA, Davidson PM, Salminen S, Thorngate III JH.** 2001. Food Additives, vol. 2nd Ed.

8. **CDC** 2012, posting date. Food Safety. [Online.]

9. **Chang Y, McLandsborough L, McClements DJ.** 2011. Interactions of a cationic antimicrobial (epsilon-polylysine) with an anionic biopolymer (pectin): an isothermal titration calorimetry, microelectrophoresis, and turbidity study. J Agric Food Chem **59:**5579-5588.

10. **Davidson PM, Sofos JN, Branen AL.** 2005. Antimicrobials in Food, 3rd ed. CRC Press, Boca Raton, FL.

11. **Helander IM, Alakomi HL, Latva-kala K, Mattila-Sanholm T, Poi I, Smid EJ, Gorris LGM, VonWright A.** 1998. Characterization of the Action of Selected Essential Oil Components on Gram-negative bacteria. Journal of Agricultural Food Chemistry **46:**3590-3595.

12. **Kurihara K, Katsuragi Y, Matsuoka I, Kashiwayanagi M, Kumazawa T, Shoji T.** 1994. Receptor mechanisms of bitter substances. Physiol Behav. **56:**1125-1132.

13. **Ma Q, Davidson PM, Zhong Q.** 2013. Antimicrobial properties of lauric arginate alone or in combination with essential oils in tryptic soy broth and 2% reduced fat milk. International Journal of Food Microbiology **166:**77-84.

14. **McClements DJ.** 2005. Food emulsions, principles, practices, and techniques. CRC Press, Boca Raton, FL.

15. **Rodriguez E.** 2004. Cellular effects of monohydrochloride of L-arginine, Nalpha-lauroyl ethylester (LAE) exposure on *Salmonella typhimurium* and *Staphylococcus aureus*. Journal of Applied Microbiology **96:**903-912.

16. **Toutain-Kidd CM, Kadivar SC, Bramante CT, Bobin SA, Zegans ME.** 2009. Polysorbate 80 inhibition of *Pseudomonas aeruginosa* biofilm formation and its cleavage by the secreted lipase LipA. Antimicrobial Agents and Chemotherapy **53:**136-145.

17. **Ziani K, Chang Y, McLandsborough L, McClements DJ.** 2011. Influence of surfactant charge on antimicrobial efficacy of surfactant-stabilized thyme oil nanoemulsions. J Agric Food Chem **59:**6247-6255.

CHAPTER V

ACCELERATED SHELF LIFE STUDIES OF LAURIC ARGINATE APPLIED SINGLY AND IN MIXED MICELLES

5.1 Abstract

The antimicrobial effects of the cationic surfactant Nα-Lauroyl-L-arginine ethyl ester monohydrochloride (lauric arginate (LAE)) applied singly or in combination with the anionic surfactant *Tween-80* were studied to compare inhibition of two foodborne pathogens (*Salmonella* sp., and *Escherichia coli* O157:H7). The influence of both exposure time and temperature upon the minimum inhibitory concentrations (MIC) was evaluated. Long term studies were conducted over the course of 32 weeks to determine the stability of the LAE inhibition of *Salmonella* sp. and *E. coli O157:H7* during storage. Within 30 seconds of exposure to LAE, an initial 2-3 log reduction was observed with all bacterial strains tested and reduced recoverable cell number remained stable over the first 5 weeks of longer term storage in the presence of LAE. However, after five weeks, increasing numbers of bacteria were recovered, although levels did not reach those of initial inoculum (10^8 CFU/mL). This increase was due to reduced effectiveness of LAE over long term storage. Results suggest that the addition of LAE to aqueous based systems can aid in the protection of microbial contamination by Gram negative bacteria during extended storage, especially when combined with *Tween-80*.

5.2 Introduction

Within the food industry, there is an ever increasing demand to improve the quality of food and ensure safety from pathogenic/spoilage microorganisms.

In the 1990's the food processing industry became much more global, and the worldwide shipment of food created a demand for the extension of shelf-life and enhanced maintenance of food quality (12). Consumer perception of a food product is the highest measure of quality. Therefore efforts of creating a safe product are greatest during the development and production process. These techniques that are implemented must last throughout the distribution and consumption stages (60). One way of measuring effectiveness is to perform shelf life studies which can provide important information to product developers.

Traditionally antimicrobials have been used to extend the shelf-life as well as inhibit spoilage microorganisms. However, in more recent years, there has been a shift in their primary usage. Antimicrobials are now being viewed as a primary mode of intervention/inactivation of pathogenic microorganisms in foods (21). Although several naturally occurring antimicrobials exist, are commercially available and applied in food processing, their efficiency, consumer acceptance, and regulation are not well defined (32).

Lauric arginate (LAE) is a cationic amphiphilic molecule made by reacting arginine with ethanol and lauric acid (10). Although not a naturally occurring antimicrobial, when ingested by humans the compound is hydrolyzed into natural components: lauric acid, L-

arginine, and ethanol (8). The antimicrobial properties of LAE are derived from its action upon the cytoplasmic membranes of microorganisms, where metabolic processes are altered yet causes no cellular lysis (58), and it has the ability to inhibit the proliferation of several microorganisms, such as bacteria, fungi, and yeasts (5, 9, 58). In 2005, the U.S. Food and Drug Administration granted Generally Recognized As Safe (GRAS) (74), however its use in industry is limited due to several reasons. One way to overcome the problems associated with instability is to complex the LAE with a co-surfactant, and create a mixed micellar systems (5).

Previous research has shown that the tendency for LAE to precipitate in aqueous solutions can be overcome by combining LAE with a non-ionic surfactant (Tween 20) to form mixed micelles (5). Micelles are aggregates of surfactant monomers in which the hydrophobic tails are assembled pointing towards the interior (away from solvent), while the hydrophilic heads orient towards the water (52). Formation of mixed micelles can also decrease the electric charge on cationic compounds decreasing their bitterness, and improving the mouthfeel (37). For this reason, the creation of antimicrobial delivery systems consisting of cationic LAE, and anionic Tween-80 (Polysorbate 80) were developed to aid in the physical stability of LAE. Tween-80 is a nonionic surfactant derived from polyoxylated sorbitol and oleic acid and is viscous but water soluble (70). Although Tween-80 possesses little antimicrobial activity alone, it has been reported to increase bacterial permeability, and enhance antimicrobial activity (70).

The growth of spoilage and pathogenic bacteria can be measured by microbiological methods, and shelf-life determinations investigating microorganisms can usually be accomplished in real time (60). In this study, we investigate the shelf life of an effective antimicrobial applied singly and in combination with a co-surfactant by challenging a broth-based system with inoculum of two foodborne pathogens (*Salmonella* sp., and *Escherichia coli* O157:H7). This is necessary to ensure the product will not only be safe for the consumer but in addition will be aesthetically pleasing at the end of its shelf life. Throughout this study we investigate the influence of time and temperature on the antimicrobial effectiveness of the created delivery systems.

5.3 Materials and methods

5.3.1 Chemicals.

Lauric Arginate (LAE) was provided by Vedeqsa Group LAMIRSA (Terrassa, Spain) under the commercial name Mirenat-N. Stock solutions were prepared at 1% (v/v) by dissolving LAE in ddH2O. *Tween-80* was purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions of *Tween-80* (1% (v/v)) were prepared by dissolving in 20mM of phosphate buffer pH 6.5. Mixed micelles were prepared by combining 25ml of 1% LAE with 50ml of 1% *Tween-80*; adjusting pH to 6.5 with HCl and filling to a final volume of 100ml with water. All other chemicals and reagents were of analytical grade supplied by Sigma Chemicals Co. (St. Louis, MO.) or Fisher Scientific (Waltham, MA). All antimicrobial solutions were filter sterilized using a 0.45-µm filter (Nalgene, Rochester, NY.) prior to use.

5.3.2 Bacterial strains and growth conditions.

Three serovars of *Salmonella enterica* (ATCC strains BAA-708 serovar Enteritidis, BAA-709 serovar Michigan, and BAA 710 serovar Montevideo) were grown in tryptic soy broth (TSB-Difco) (32°C), and one strain of *Escherichia coli* O157:H7 (ATCC 43895) was Luria Bertani broth (LB-Difco) (37°C). Stock cultures of all organisms were kept at -80°C in 25% glycerol. Working cultures were streaked on either trypic soy agar (TSA, *Salmonella*) or Luria Bertani agar (LBA) plates (*E. coli* O157:H7) wrapped in parafilm and stored at 4°C for 4 weeks. For experimental purposes, all organisms were grown overnight and OD_{600} adjusted to 0.1 ($\approx 10^8$ CFU/mL) prior to experimentation.

5.3.3 Determination of minimum inhibitory concentration (MIC).

The MIC was determined by plate enumeration TSB (*Salmonella*) or LB (*E. coli* O157:H7). Working solutions of the antimicrobial or surfactant combination were prepared by diluting the 1% stock solutions in TSB or LB to produce final LAE concentrations of 40-110 ppm. An overnight sample of the bacterial culture was diluted to approximately 10^8 CFU/ml in TSB or LB in phosphate buffered saline (Dissolve 8g of NaCl, 0.2g of KCl, 1.44g of Na2HPO4, and 0.24 g of KH2PO4 in 800 ml of distilled H20. Adjust the pH to 7.4 with HCl. Add H2O to 1L. Autoclave for 20mins. (Source: Molecular Cloning 2nd Ed. page B.12)) and 1% inoculum transferred to test tubes, produce an initial cell concentration of approximately 10^6 CFU/ml. After exposure for 30s, 24hr, 48hr, 72hr, and 96hr, 50µl samples were removed from test tubes, diluted in PBS, and plated on TSA or LB agar using AUTOPLATE spiral plater (Advanced

Instruments INC., Norwood, MA). After incubation at 32°C (*Salmonella*) or 37°C (*E. coli* O157:H7) for 48h, colonies were counted using the SCAN 500 (Interscience, France). The MIC was defined as the lowest concentration of antimicrobial agent required to inhibit development of visible growth after 24hr of incubation. The minimum detection limit was 2.0×10^1 CFU/ml.

5.3.4 Shelf life studies.

To test the antimicrobial effectiveness of LAE alone, or LAE complexed with Tween-80, inoculated samples of each were prepared at the MIC and stored at 32°C (*Salmonella*) or 37°C (E. coli O157:H7). Samples were taken weekly for 12 weeks, then every 10 weeks thereafter for a total of 32 weeks. Samples were diluted in PBS, and plated using the AUTOPLATE spiral plater. Samples were incubated, and CFU/ml determined by using the SCAN 500. Log reductions (LR) at each time point were calculated as follows: LR = Initial level of bacteria inoculated into the antimicrobial at day 0 (log CFU/ml) – surviving number of bacteria (log CFU/ml) at a given time.

5.3.5 Chemical stability during long term storage.

To test the stability and effectiveness of LAE alone or complexed with Tween-80, uninoculated samples of each were prepared at the MIC and stored in LB medium at 37°C. Broth samples were removed weekly (for a total of 8 weeks). The antimicrobial effectiveness of the stored LAE solution was evaluated through the addition of E.coli O157:H7 culture and surviving cell numbers were enumerated after exposure times of 30s, 1hr, and 5hr.

Statistical analysis Statistical Analysis System (SAS) software was used to determine statistical significance using ANOVA.

5.4 Results

5.4.1 Physical stability studies

Although active across a wide pH range (3-7), precipitation of LAE from solution has been reported at pH > 4.5 (4). Figure 4.1a demonstrates the tendency for LAE to precipitate from solution when combined with phosphate buffer pH 6.5. After 24 hours of storage at room temperature, large colloidal aggregates were observed, and to prevent any issues with stability, 0.25% LAE was combined with varying concentrations of a co-surfactant (Tween-80) and stored at room temperature for 24h until a physically stable system was formed. Results from this experiment are presented in figure 4.1b, and reveal that the best combination (indicated my no physical turbidity) was achieved when 0.25% LAE was combined with 0.5% Tween-80 (v/v) in phosphate buffer pH 6.5.

5.4.2 Minimum Inhibitory Concentration

MICs for LAE alone and in combination with Tween-80 are provided in Table 4.1. Exposure to LAE alone resulted in inhibition of *E. coli* and all three serovars of *S. enterica* at 50ppm; when combined with Tween-80 inhibition was observed at 100ppm. Although the MIC for LAE:Tween-80 is twice the value of that when LAE is applied singly, the overall concentration of LAE is the same in both solutions. Therefore, the

presence of Tween-80 does not change or enhance the antimicrobial efficacy of LAE, however the presence of *Tween-80* prevents the flocculation of the antimicrobial.

5.4.3 Antimicrobial efficiency during extended storage

MIC values determined through previous studies were utilized in these experiments, and antimicrobial efficacy tested during extended storage at elevated temperatures. Figures 5.1-5.4 show the change in population of *E. coli* O157:H7 and *S. enterica sp.* during a 32 week trial in broth at 37°C and 32°C respectively. Samples were initially inoculated with log 7 CFU/ml and 50ppm LAE or 100ppm LAE:Tween-80. After 1 week of storage, population levels reveal susceptibility to antimicrobial treatment and large reductions in cell survival was observed falling at or below the limit of detection. This trend was observed for the first 4 weeks of storage, and during the remaining weeks of storage cell levels began to increase but did not reach those of the initial inoculum.

5.4.4 Investigating chemical stability of LAE & LAE:Tween-80 during extended storage

In order to determine if the regrowth observed at week 5 during the extended storage trial was the result of chemical instability, uninoculated samples of LAE (50ppm) and LAE:Tween-80 (100ppm) stock solutions were prepared and stored at 37°C. On day zero and every two weeks thereafter for a total of eight weeks, 5 ml of solution was removed and inoculated with overnight samples of *E. coli* O157:H7. Levels of surviving cells were monitored at 30 sec, 1h and 5h post inoculation. After six weeks of storage, the rate of microbial destruction was observed to decrease (Figure 5.5), indicating that the

antimicrobial efficacy upon storage was gradually decreasing over time. Eight weeks of storage appears to have caused a reduction in the antimicrobial effectiveness of LAE alone. Samples applied to an overnight culture of *E. coli* O157:H7 show an initial reduction of 1.5 log CFU/ml. When samples were taken at 1h and 5h no reduction was observed and cell levels reached those of the initial inoculum indicating storage time and temperature has an antagonistic effect on the inhibitory effects of LAE. However, LAE:Tween-80 maintained its inhibitory effects with an initial 1.5 log CFU/ml reduction after 30s of exposure, and close to a 3.5 log reduction after exposure for both 1 and 5h. This finding indicates that the long term functionality of LAE is enhanced and maintained when combined with a co-surfactant.

5.4.5 Statistical significance.

Tables 5.1-5.5 reveal significant ($P<0.05$) interactions among concentrations of LAE and LAE:Tween-80 during long term storage. Significance is strain dependent.

5.5 Discussion

Antimicrobials are often added to unprocessed/processed foods in attempt to extend shelf life by reducing the risk of microbial contamination. Our studies have investigated the effects of both time and temperature on the antimicrobial effectiveness of the GRAS antimicrobial LAE applied singly (50ppm) or in combination with Tween-80 (100ppm) These studies were performed during long term storage (32 weeks (224 days)), and in appropriate growth media. When combinations of antimicrobials are used, interactions amongst the combinations can be synergistic, additive, or antagonistic (11), and previous

research from our laboratory has shown that synergistic activity of LAE is achieved when combined with a co-surfactant, Tween-80, resulting in greater functionality. In 2011, Asker *et al.* also showed that the creation of mixed micelles resulted in increased stability of LAE in solutions whose pH exceeded 4.5 (4).

The primary objective of this study was to establish if LAE when applied alone or in combination would inhibit outgrowth of *E. coli* 0157:H7 and *S enterica* during extended shelf life. In shelf life studies using these gram-negative organisms at an initial inoculum of ~ 8 log CFU/ml, a two log reduction of pathogen levels were observed after 30 seconds of exposure to LAE and LAE:Tween-80. After one week of storage at 37°C, cell levels of *E. coli* 0157:H7 dropped below the minimal level of detection, and this trend was observed for the first four weeks of treatment with LAE, and the first two weeks when treated with LAE: *Tween-80*. Differences in inhibition were observed amongst all four gram-negative organisms tested indicating the effectiveness of LAE and LAE: *Tween-80* is strain specific (table 5.1-5.4). After week four, an increase in pathogen survival was observed and continued for the duration of the study, however levels did not reach those of the initial inoculum. Similar to findings reported by Luchansky *et al*, when LAE is incubated and inoculated for an extended period of time, the inhibitory efficacy becomes compromised (43). After 60 days of storage at 4°C, Luchansky observed a 2 log increase in cell levels of *L.monocytogenes* when treated with 5% LAE on the surface of ham.

Holly *et al* reported that food grade chemicals have been added during food manufacture to extend shelf life by stabilizing chemical change or by preventing or inhibiting microbial growth (28). The evident outgrowth both *E. coli* O157:H7 and *Salmonella* sp. brought into question the chemical stability of LAE during long term storage. Microbial adaptation was a possibility for the outgrowth observed after four weeks of storage. For this reason, transfer studies were performed (data not shown) in which samples of *E. coli* 0157:H7 exposed to LAE for 32 weeks were inoculated into a freshly prepared solution of LAE at a concentration of 45 ppm (below the MIC). Samples were transferred to a fresh solution of LAE every 24 hours for a total of 72 hours, and plated after 72 hours to investigate cell survival. After 72 hours of exposure cell numbers were higher than the initial inoculum indicating no adaptation had occurred.

We investigated the antimicrobial efficacy of LAE and LAE: *Tween-80* under accelerated storage conditions by preparing stock solutions at the determined MICs and storing at 37°C uninoculated. Samples were taken every two weeks for a total of eight weeks and applied to an overnight culture of *E. coli* 0157 H7. After four weeks of storage, inhibitory abilities appeared to have diminished evident by increased levels of survival (figure 5.5). After eight weeks of storage cell levels reached those of the initial inoculum for the LAE stock solution. These findings lead us to the conclusion that both time and temperature has an adverse effect on the antimicrobial efficiency of both LAE and LAE: *Tween-80*; although solutions of LAE: *Tween-80* exhibited lower levels of cell survival.

Throughout these studies we have validated the inhibitory effects of LAE applied singly or in combination with Tween-80 against two Gram-negative pathogens. We found Tween-80 enhances the activity of LAE against *E. coli* O157:H7 and *Salmonella* sp. in microbiological media during extended storage. Other researchers have also reported that treatment with LAE will decrease the levels and prevent the outgrowth of several foodborne pathogens making it ideal as an additive to prevent microbial contamination. The outgrowth of parent after four weeks of storage demonstrates that antimicrobial activity in food systems could be affected by several factors including pH, time, and storage temperature.

Table 4.1[1]Bacterial strains were inoculated with varying amounts of LAE alone, or LAE:Tween-80 (mixed micelle), incubated at 32°C or 37°C, and samples taken every 24 hours for 96 hours. Cell survival was determined by plate enumeration.

Bacterium	Isolate	LAE MIC[1] (ppm)	LAE:Tween-80 MIC[1] (ppm)
Gram negative			
Salmonella enterica serovar Enteritidis	ATCC BAA-708	50	100
Salmonella enterica serovar Michigan	ATCC BAA-709	50	100
Salmonella enterica serovar Montevideo	ATCC BAA-710	50	100
Escherichia coli O157:H7	ATCC 43895	50	100
Gram positive			
Listeria monocytogenes (LM 9)	CU DD6824	18	40
Listeria monocytogenes (LM 10)	CU FSL-N1-304	16	40
Listeria monocytogenes (LM 21-Scott A)	CU FSL-J1-225	18	40

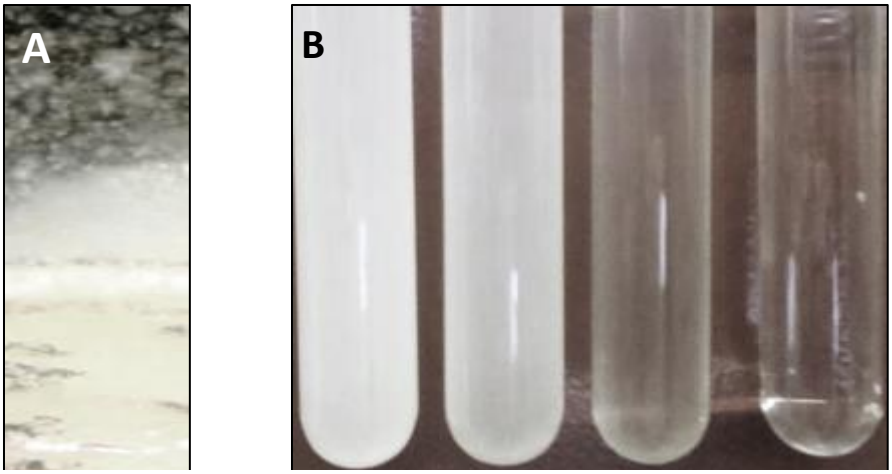


Figure 4.1. Visual appearance of aqueous solutions containing a)LAE alone and b) LAE:*Tween-80* complexes (pH 6.5) with 0.25% LAE and varying ratios of Tween-80 as indicated

Table 5. 1 Cell survival after treatment of LAE applied singly (50 ppm) or in combination with Tween-80 (100 ppm) to control *E. coli* O157:H7 during long-term storage at 37°C

	0	1	2	3	4	5	9	10	11	12	22	32
E. coli O157:H7 control	7.61	9.07	8.53	8.68	8.43	7.70	7.65	6.78	7.07	7.34	5.94	6.30
LAE[a]	5.20	1.30*	1.30*	1.30*	1.30*	1.72	2.32	3.12	3.59	2.57	2.85	1.30*
LAE:Tween-80[a]	5.77	1.30*	1.30*	2.69	3.44	3.18	2.96	1.30*	3.03	3.40	5.42	2.27

* Minimum detection level (1.30 $\log_{10}$ CFU/ml)

[a] Values with the same letter are statistically similar.

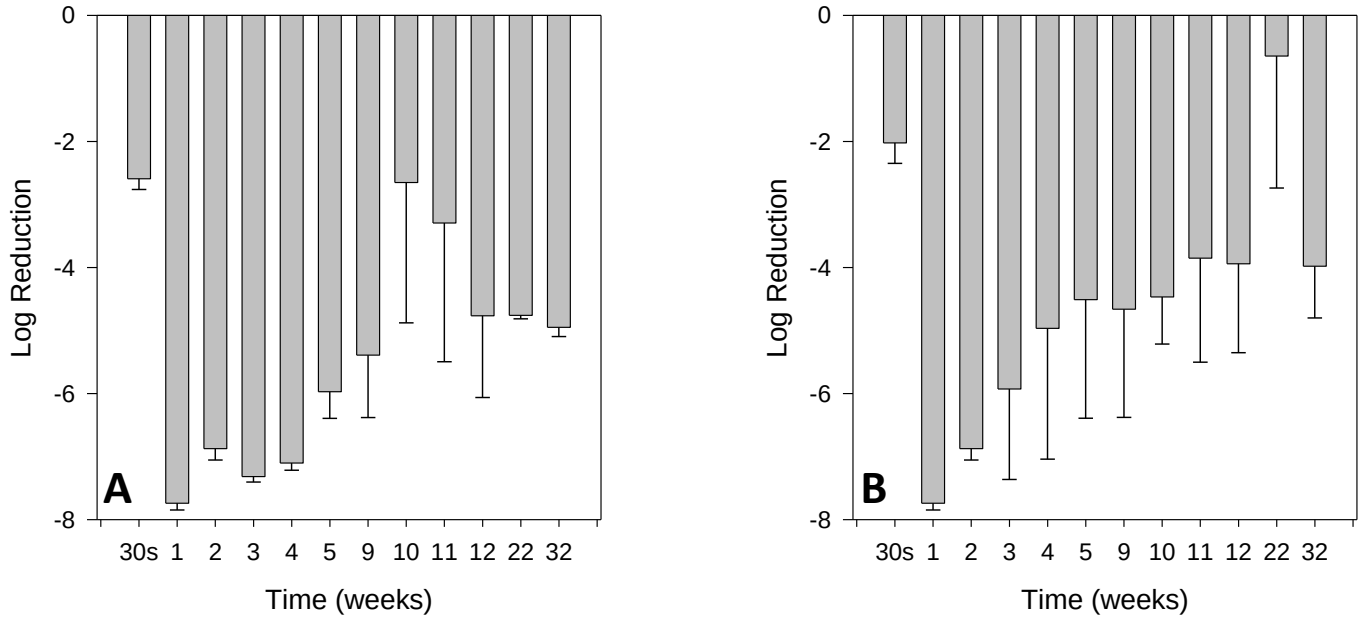


Figure 5. 1 Quantification of antimicrobial efficiency of A) LAE or B) LAE:Tween-80 on *E. coli* O157:H7 during extended storage at 37°C. Error bars represent standard error of the mean.

Table 5. 2 Cell survival after treatment of LAE applied singly (50 ppm) or in combination with Tween-80 (100ppm) to control *S. enterica* Enteritidis during long-term storage at 32°C. Values reported as Cell survival Log CFU/ml.

Samples	Storage time (weeks)											
	0	1	2	3	4	5	9	10	11	12	22	32
S. enterica Enteritidis control	7.61	9.07	8.53	8.68	8.43	7.70	7.65	6.78	7.07	7.34	5.94	6.30
LAE	5.74	1.30*	1.30*	1.84	2.10	1.30*	1.30*	1.30*	1.30*	4.22	6.55	3.74
LAE:Tween-80	5.40	2.44	2.63	2.58	1.30*	2.86	3.10	3.63	6.11	5.78	5.47	6.79

* Minimum detection level (1.30 $\log_{10}$ CFU/ml)

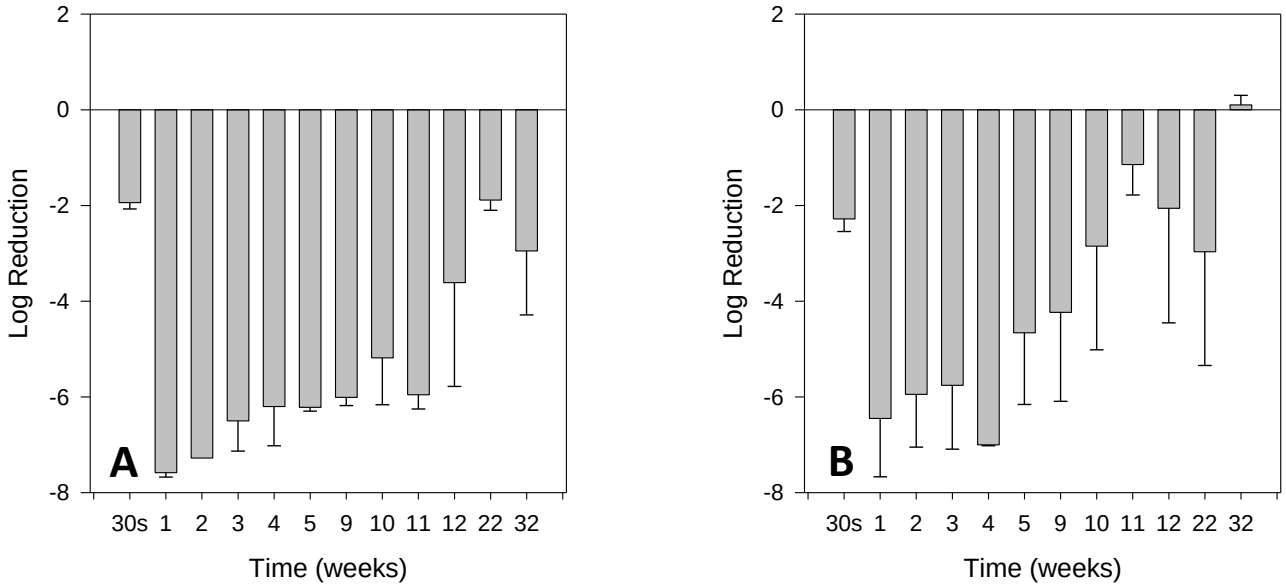


Figure 5. 2 Quantification of antimicrobial efficiency of A) LAE or B) LAE:Tween-80 on *Salmonella* Enteritidis during extended storage at 32°C. Error bars represent standard error of the mean.

Table 5. 3 Cell survival after treatment of LAE applied singly (50 ppm) or in combination with Tween-80 (100 ppm) to control *S. enterica* Michigan during long-term storage at 32°C. Values reported as Cell survival Log CFU/ml.

Samples	Storage time (weeks)											
	0	1	2	3	4	5	9	10	11	12	22	32
S. enterica Michighan control	7.61	9.07	8.53	8.68	8.43	7.70	7.65	6.78	7.07	7.34	5.94	6.30
LAE	5.62	2.57	1.30*	3.90	5.07	3.93	3.08	4.57	3.99	5.88	7.28	5.79
LAE:Tween-80	4.07	1.30*	1.85	2.02	4.15	3.23	1.30*	1.30*	2.69	3.47	5.37	4.08

* Minimum detection level (1.30 $\log_{10}$ CFU/ml)

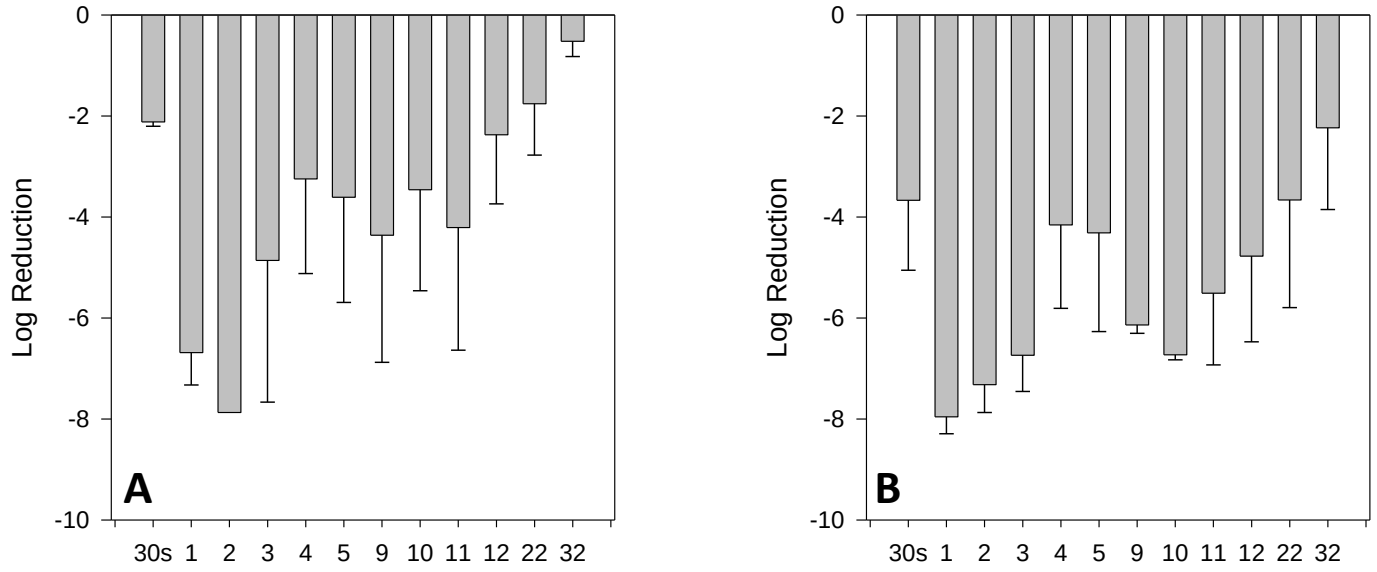


Figure 5. 3 Quantification of antimicrobial efficiency of A) LAE or B) LAE:Tween-80 on *Salmonella* Michigan during extended storage at 32°C. Efficiency determined by log reduction; error bars represent standard error of the mean

Table 5. 4 Cell survival after treatment of LAE applied singly (50 ppm) or in combination with Tween-80 (100 ppm) to control *S. enterica* Montevideo during long-term storage at 32°C. Values reported as Cell survival Log CFU/ml.

Samples	Storage time (weeks)											
	0	1	2	3	4	5	9	10	11	12	22	32
S. enterica Montevideo control	7.61	9.07	8.53	8.68	8.43	7.70	7.65	6.78	7.07	7.34	5.94	6.30
LAE	5.64	1.88	1.89	2.88	3.29	4.81	5.11	3.47	5.17	5.75	5.75	6.38
LAE:Tween-80	2.94	2.25	1.85	2.08	3.38	3.18	3.13	1.30*	3.21	2.82	5.59	5.45

* Minimum detection level (1.30 $\log_{10}$ CFU/ml)

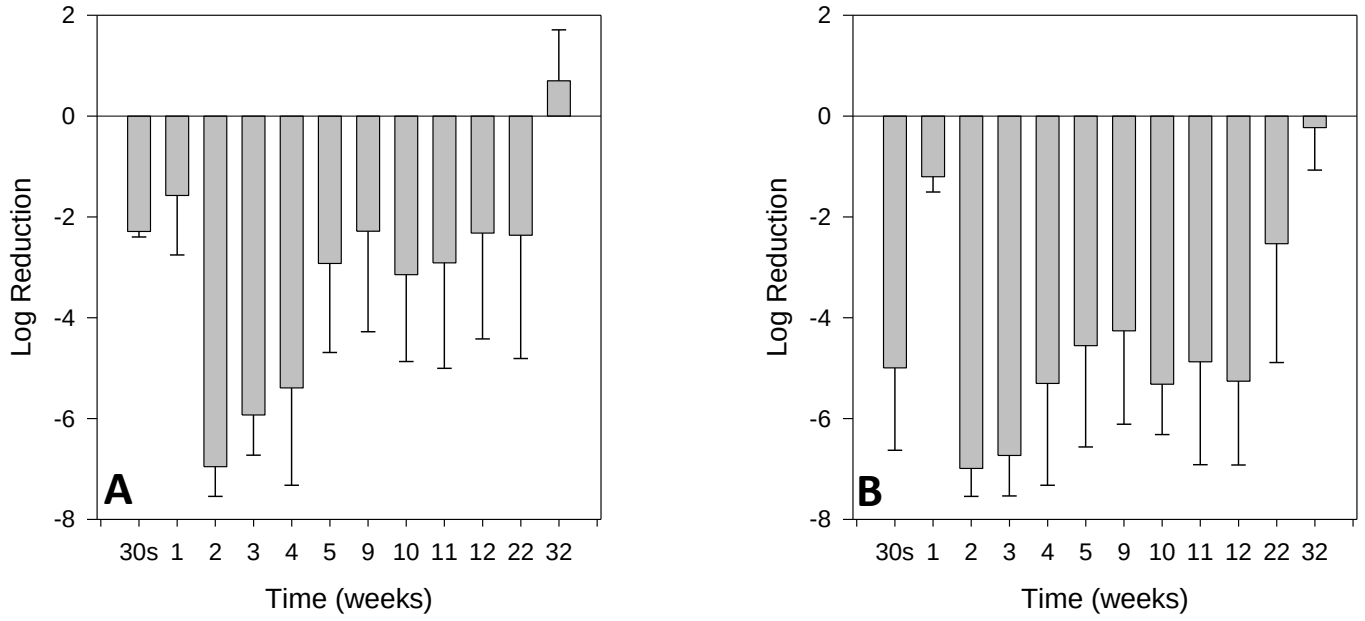


Figure 5. 4 Quantification of antimicrobial efficiency of A) LAE or B) LAE:Tween-80 on *Salmonella* Montevideo during extended storage at 32°C. Efficiency determined by log reduction; error bars represent standard error of the mean

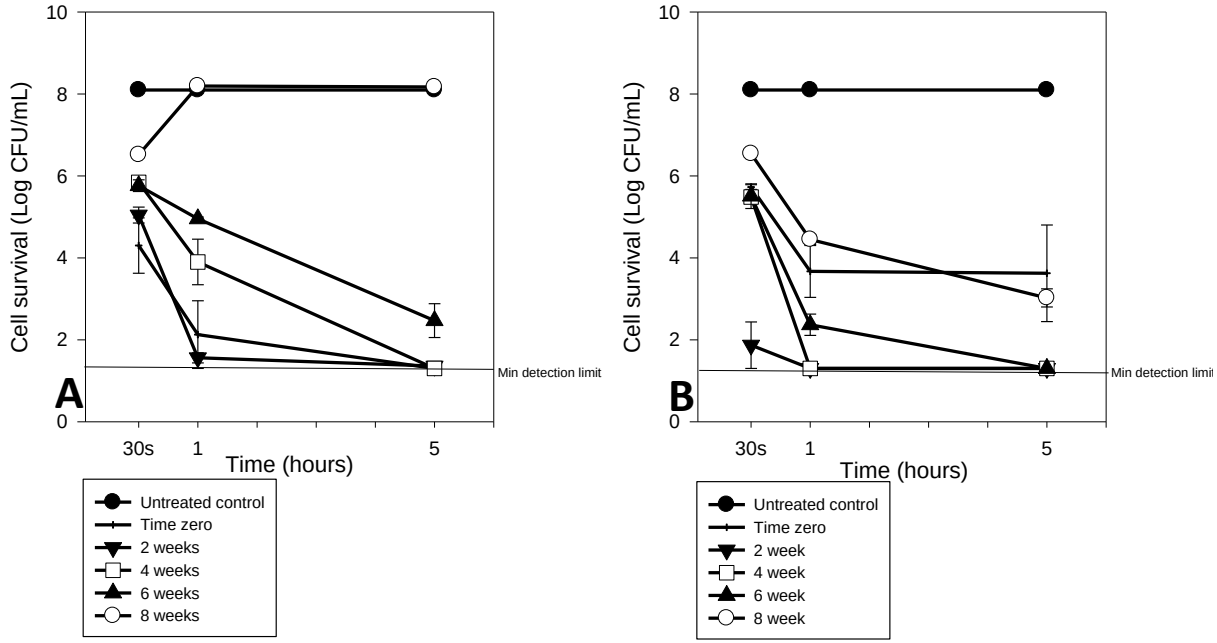


Figure 5. 5 Samples of LAE and LAE:Tween-80 (M.I.C) in Luria Bertani broth and stored uninoculated at 37°C. Every 2 weeks, samples of antimicrobials were removed and inoculated with overnight samples of *E. coli* O157:H7. Surviving cell numbers were measured just after inoculation (30 seconds) and after 1, and 5 hour(s) of exposure

Table 5. 5 Cell survival in Log CFU/ml after treatment with LAE and LAE:Tween-80 stored at 37C for 8 weeks. Samples were inoculated with overnight culture of *E. coli* O157:H7 every 2 weeks and cell survival monitored after 30s, 1h, and 5h of exposure.

LAE 50ppm	Storage time (weeks) 37°C				
	0	2	4	6	8
Control (untreated)	8.10				
30 sec	4.30	5.04	5.84	5.76	6.52
1 hour	2.13	1.56	3.90	4.95	8.19
5 hours	1.30*	1.36	1.30*	2.47	8.17

* Minimum detection level (1.30 $\log_{10}$ CFU/ml)

LAE:Tween-80 100ppm	Storage time (weeks) 37°C				
	0	2	4	6	8
Control (untreated)	8.10				
30 sec	5.73	1.87	5.84	5.76	5.51
1 hour	3.67	1.30*	3.90	4.95	2.37
5 hours	3.62	1.30*	1.30*	2.47	1.30*

* Minimum detection level (1.30 $\log_{10}$ CFU/ml)

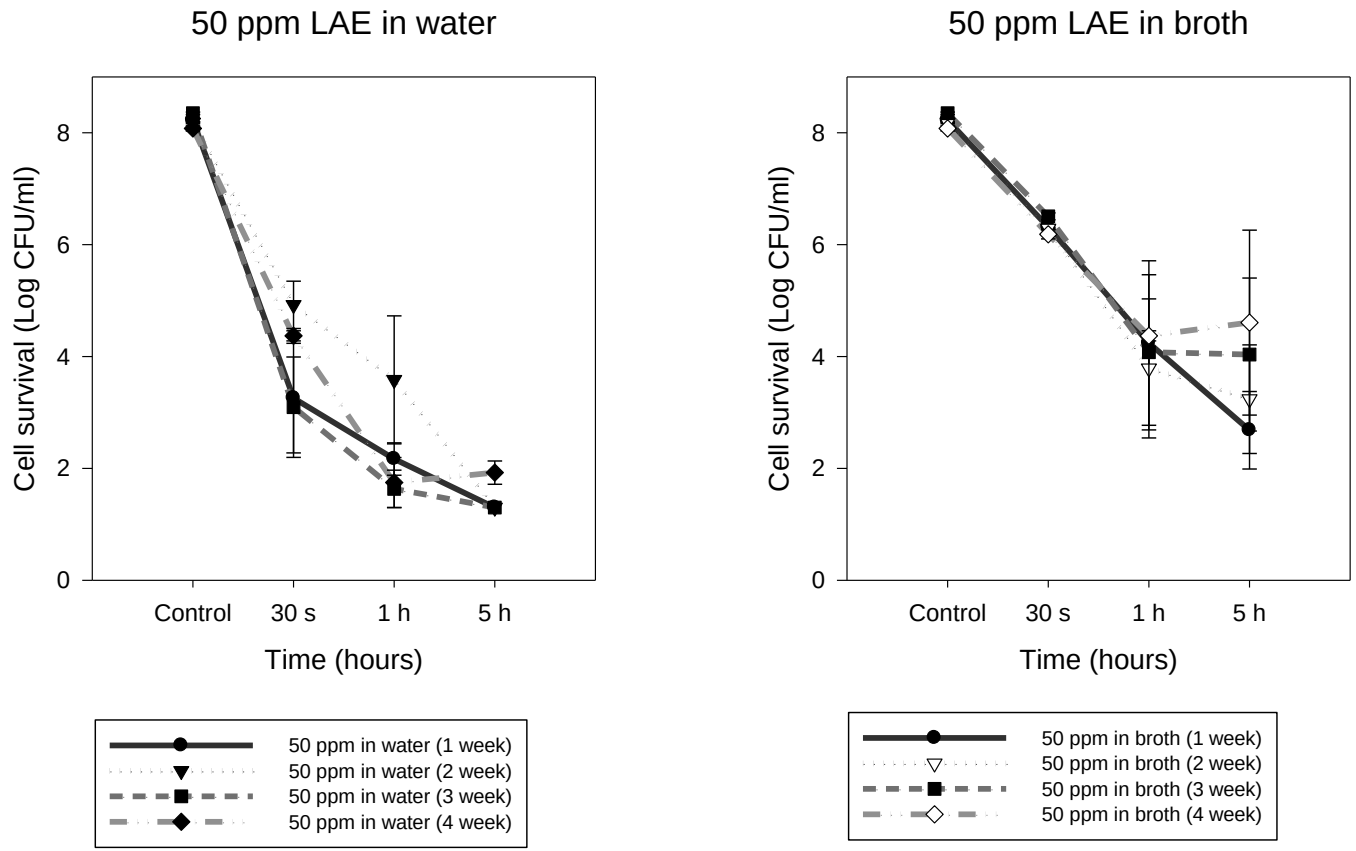


Figure 5. 6 Samples of LAE (M.I.C) in water or Luria Bertani broth and stored uninoculated at 37°C. Every week, samples of antimicrobials were removed and inoculated with overnight cultures of *E. coli* O157:H7. Surviving cell numbers were measured just after inoculation (30 seconds) and after 1, and 5 hour(s) of exposure

Table 5. 6 Statistical analysis via a two-way ANOVA comparing effectiveness of LAE on *E. coli* O157:H7 during long term storage at 37°C. Bonferonni test was used to determine significance.

Time 0 vs. Week 2

Control	P> 0.05	ns
30s	P> 0.05	ns
1hr	P> 0.05	ns
5hr	P> 0.05	ns

Time 0 vs. Week 4

Control	P> 0.05	ns
30s	P< 0.01	s
1hr	P< 0.001	s
5hr	P> 0.05	ns

Time 0 vs. Week 6

Control	P> 0.05	ns
30s	P< 0.01	s
1hr	P< 0.001	s
5hr	P< 0.05	s

Time 0 vs. Week 8

Control	P> 0.05	ns
30s	P< 0.001	s
1hr	P< 0.001	s
5hr	P< 0.001	s

Week 2 vs. Week 4

Control	P> 0.05	ns
30s	P> 0.05	ns
1hr	P< 0.001	s
5hr	P> 0.05	ns

Week 2 vs. Week 6

Control	P> 0.05	ns
30s	P> 0.05	ns
1hr	P< 0.001	s
5hr	P< 0.05	s

Week 2 vs. Week 8

Control	P> 0.05	ns
30s	P< 0.01	s
1hr	P< 0.001	s
5hr	P< 0.001	s

Week 4 vs. Week 6

Control	P> 0.05	ns
30s	P> 0.05	ns
1hr	P> 0.05	ns
5hr	P< 0.05	s

Week 4 vs. Week 8

Control	P> 0.05	ns
30s	P> 0.05	ns
1hr	P< 0.001	s
5hr	P< 0.001	s

Week 6 vs. Week 8

Control	P> 0.05	ns
30s	P> 0.05	ns
1hr	P< 0.001	s
5hr	P< 0.001	s

Table 5. 7 Statistical analysis via a two-way ANOVA comparing effectiveness of LAE:Tween-80 on *E. coli* O157:H7 during long term storage at 37°C. Bonferonni test was used to determine significance.

Time 0 vs. Week 2

Control	P> 0.05	ns
30s	P< 0.001	s
1hr	P< 0.001	s
5hr	P< 0.001	s

Week 2 vs. Week 4

Control	P> 0.05	Ns
30s	P< 0.001	S
1hr	P> 0.05	Ns
5hr	P> 0.05	ns

Week 4 vs. Week 8

Control	P> 0.05	ns
30s	P> 0.05	ns
1hr	P< 0.001	s
5hr	P< 0.01	s

Time 0 vs. Week 4

Control	P> 0.05	ns
30s	P> 0.05	ns
1hr	P< 0.001	s
5hr	P< 0.001	s

Week 2 vs. Week 6

Control	P> 0.05	ns
30s	P< 0.001	s
1hr	P> 0.05	ns
5hr	P> 0.05	ns

Week 6 vs. Week 8

Control	P> 0.05	ns
30s	P> 0.05	ns
1hr	P< 0.001	s
5hr	P< 0.01	s

Time 0 vs. Week 6

Control	P> 0.05	ns
30s	P> 0.05	ns
1hr	P< 0.05	s
5hr	P< 0.001	s

Week 2 vs. Week 8

Control	P> 0.05	ns
30s	P< 0.001	s
1hr	P< 0.001	s
5hr	P< 0.01	s

Time 0 vs. Week 8

Control	P> 0.05	ns
30s	P> 0.05	ns
1hr	P> 0.05	ns
5hr	P> 0.05	ns

Week 4 vs. Week 6

Control	P> 0.05	ns
30s	P> 0.05	ns
1hr	P> 0.05	ns
5hr	P> 0.05	ns

References

1. **Asker D, Weiss J, McClements DJ.** 2011. Formation and stabilization of antimicrobial delivery systems based on electrostatic complexes of cationic-non-ionic mixed micelles and anionic polysaccharides. J Agric Food Chem **59:**1041-1049.

2. **Asker D, Weiss, J., McClements, D.** 2008. Analysis of the interactions of a cationic surfactant (lauric arginate) with an anionic bioplymer (pectin): isothermal titration calorimetry, light scattering, and microelectrophoresis. Langmuir **25:**116-122.

3. **Bonnaud M, Weiss J, McClements DJ.** 2010. Interaction of a food-grade cationic surfactant (lauric arginate) with food-grade biopolymers (pectin, carrageenan, xanthan, alginate, dextran, and chitosan). J Agric Food Chem **58:**9770-9777.

4. **Bonnaud M, Weiss, J., McClements, D.J.** 2010. Interaction of a food-grade cationic surfactant (lauric arginate) with food-grade biopolymers (pectin, carrageenan, xanhan, alginate, dextran, and chitosan). J Agr Food Chem **58:**9770-9777.

5. **Brandt AL, Castillo A, Harris KB, Keeton JT, Hardin MD, Taylor TM.** 2010. Inhibition of *Listeria monocytogenes* by food antimicrobials applied singly and in combination. J Food Sci **75:**M557-563.

6. **Branen JK, Davidson PM.** 2004. Enhancement of nisin, lysozyme, and monolaurin antimicrobial activities by ethylenediaminetetraacetic acid and lactoferrin. International Journal of Food Microbiology **90:**63-74.

7. **Branen LA, Davidson PM, Salminen S, Thorngate III JH.** 2001. Food Additives, 2nd Ed.

8. **Davidson PM, Sofos JN, Branen AL.** 2005. Antimicrobials in Food, 3rd ed. CRC Press, Boca Raton, FL.

9. **Holley RA, Patel D.** 2005. Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. Food Microbiology **22:**273-292.

10. **Juneja VK, Dwivedi HP, Yan X.** 2012. Novel Natural Food Antimicrobials*. Annual Review of Food Science and Technology **3:**381-403.

11. **Kurihara K, Katsuragi Y, Matsuoka I, Kashiwayanagi M, Kumazawa T, Shoji T.** 1994. Receptor mechanisms of bitter substances. Physiol Behav. **56:**1125-1132.

12. **Luchansky JB, Call JE, Hristova B, Rumery L, Yoder L, Oser A.** 2005. Viability of *Listeria monocytogenes* on commercially - prepared hams surface treated with acidic calcium sulfte and lauric arginate and stored at 4^0C. Meat Sci **71:**92-99.

13. **McClements DJ.** 2005. Food emulsions, principles, practices, and techniques. CRC Press, Boca Raton, FL.

14. **Rodriguez E.** 2004. Cellular effects of monohydrochloride of L-arginine, Nalpha-lauroyl ethylester (LAE) exposure on *Salmonella typhimurium* and *Staphylococcus aureus*. Journal of Applied Microbiology **96:**903-912.

15. **Sewald M, Jon DeVries.** Food Product Shelf -Life. *In Laboratories*, Medallion Laboratories.

16. **Toutain-Kidd CM, Kadivar SC, Bramante CT, Bobin SA, Zegans ME.** 2009. Polysorbate 80 inhibition of *Pseudomonas aeruginosa* biofilm formation and its cleavage by the secreted lipase LipA. Antimicrobial Agents and Chemotherapy **53:**136-145.

17. **USDA** 2005, Agency Response Letter GRAS Notice No. GRN 000164. [Online.]

CHAPTER VI

INVESTIGATING THE MECHANISTIC TARGET(S) OF LAURIC ARGINATE AGAINST *LISTERIA MONOCYTOGENES* AND *SALMONELLA ENTERICA*

6.1 Abstract

The antimicrobial surfactant lauric arginate (LAE) possesses activity against a wide range of microorganisms including bacteria, yeast, and fungi, however its mechanisms of action remain ambiguous. Grown in either TSB-YE, or TSB at 32°C, lauric arginate was found to be effective at preventing the growth of *L monocytogenes* and *S enterica* at 18 ppm and 50 ppm respectively. Mixed micellar systems were created to reduce the rate of precipitation of lauric arginate from solution by combining LAE with the co surfactant *Tween-80*. The MIC for mixed micelles upon the test of microorganisms was 40 ppm and 100 ppm. To investigate the mechanistic target of lauric arginate analysis of disruption of membrane potential ($\Delta\Psi$) using the proton ionophore valinomycin via flow cytometry was performed. Disruption of membrane potential as indicated by hyperpolarization after exposure to antimicrobial treatment was observed in samples exposed to LAE, LAE:Tween-80, but not Tween-80 leading to the conclusion that LAE does target the membrane potential within the cytoplasm.

6.2 Introduction

Antimicrobials have long been used to reduce the proliferation of microorganisms, and both the antifungal and antibacterial properties have been investigated and described in several studies (21, 25, 45, 58, 66, 82). Surfactants are a special class of antimicrobials whose chemical properties lead to their antimicrobial activity, and whose actions may degrade or solubilize cell membranes at concentrations even below critical micellar concentration (CMC). Application of these antimicrobial agents can lead to loses of membrane potential, alteration of cell permeability and leakage of ions, resulting in alterations in metabolic inhibition, growth arrest or cell lysis (33).

LAE is effective at inhibiting the proliferation of several microorganisms; including bacteria, fungi, and yeasts (31), yet not much is known about the mechanisms of action of this antimicrobial. Research presented by Rodriguez *et al* implicate the antimicrobial properties of LAE disrupt the cytoplasmic membranes of microorganisms, and supported by use of electron microscopy, this disruption/instability of the plasma membrane lipid bilayer is thought to alter the metabolic process and hinder the cellular cycle, all without causing cellular lysis. (7, 58). Observations using electronic transmitting microscopy have been reported, and confirm alterations caused by LAE on the bacteria cytoplasmic membrane (58). However, to date there are no known studies that specifically investigate the mechanisms of action. Several approaches have been developed to investigate the mode of action of cationic compounds; such processes include potential membrane disturbance, alteration of the efflux pumps, leakage of cytoplasm constituents or structural changes (56, 64, 67). Rodrigues *et al* indicated

alterations in cell integrity (mainly in the outer membrane), but no significant change in the cytoplasm when LAE was applied to *S. typhimurium* were observed. When applied to *S. aureus*, clear zones surrounding the inner membrane, abnormal septation and mesosome-like structures (folds in the plasma membrane) were observed in the cytoplasm. By measuring the flow of potassium across the membrane, this research group has reported the induction of transmembrane ion flux due to treatment with LAE.

Several researchers have reported methods which measure the effects of antimicrobials/essential oils on the influence on biological membranes (25, 71). The cytoplasmic membrane of bacteria has two principal functions: (i) barrier function and energy transduction, which allow the membrane to form ion gradients that can be used to drive various processes, and (ii) formation of a matrix for membrane-embedded proteins (such as the membrane-integrated F_0 complex of ATP-synthase) (27). Changes in the energy-transducing process have been studied by monitoring effects on the intracellular ATP pool, the membrane potential, the pH gradient across the cytoplasmic membrane, and the potassium gradient (13).

Studies using a well know essential oil, carvacrol, have reported similar findings and results that show increases in the membrane fluidity and leakage of protons and potassium ions, leading to a decrease in the pH gradient across the cytoplasmic membrane (Δ pH), a collapse of the membrane potential ($\Delta\Psi$), and the inhibition of ATP synthesis. Finally, these events are followed by cell death (71).

A substantial amount of data indicates that most antimicrobial peptides interact with the cytoplasmic membrane rather than by interacting with a specific protein receptor (80). Davidson *et al* has previously reported that two ways of examining the mechanisms

of action of antimicrobial agents exist. The first approach is to consider a range of compounds individually, and the second investigates possible targets (cell wall, cytoplasmic membrane, outer membrane, inner membrane, functional and structural proteins, enzymes, DNA, and RNA) within microbial cells. Investigation of these targets later reveal how antimicrobials interact with them, and the effects they have; if any (21). Rodriguez *et al* have reported that treatment with LAE results in disruption of the cellular cycle yet causes no lysis (58). Although no lysis was observed, damage to the cytoplasmic membrane can occur in several ways: leakage, lysis, enzyme inhibition, and dissipation of the proton motive force (PMF) (21). The release of intracellular constituents is usually the result of damage to the cytoplasmic membrane; the first being potassium (K^+).

The PMF is a mechanism by which gradients move across the cytoplasmic membrane, and power bacterial cells by active transport, oxidative phosphorylation, and adenosine triphosphate (ATP). Treatment with antimicrobials can inhibit active transport and also halt transmembrane flux thus affecting membrane integrity (21).

The aim of the work presented here was to identify potential targets of the bacterial cell, and the response to antimicrobial treatment. More than one mechanistic target may be involved in the inhibitory abilities of LAE, therefore we investigated the disruption of membrane potential, and depolarization or hyperpolarization at bactericidal concentrations of LAE, LAE: tween-80, and concentrations of Tween-80 at levels used in the formation of mixed micellar systems. Flow cytometry was the primary tools of investigation.

6.3 Materials and methods

6.3.1 Chemicals.

Lauric Arginate (LAE) was provided by Vedeqsa Group LAMIRSA (Terrassa, Spain) under the commercial name Mirenat-N. Stock solutions were prepared at 1% (v/v) by dissolving LAE in ddH2O. Tween-80 was purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions of *Tween-80* (1% (v/v)) were prepared by dissolving in 20mM of phosphate buffer pH 6.5. Mixed micelles were prepared by combining 25ml of 1% LAE with 50ml of 1% *Tween-80*; adjusting pH to 6.5 with HCl and filling to a final volume of 100ml with water. BacLight™ Bacterial Membrane Potential Kit (B34950) was purchased from Molecular Probes (Eugene, OR.). All other chemicals and reagents were of analytical grade supplied by Sigma Chemicals Co. (St. Louis, MO.) or Fisher Scientific (Waltham, MA) All antimicrobial solutions were filter sterilized using a 0.45-µm filter (Nalgene, Rochester, NY.) prior to use.

6.3.2 Bacterial strains and growth conditions.

Salmonella enterica (ATCC BAA-708 serovar Enteritidis) was grown in tryptic soy broth (TSB-Difco) (32°C), *Escherichia coli* O157:H7 (ATCC 43895) was grown in Luria Bertani broth (LB-Difco) (37°C), and *Listeria monocytogenes* (CU FSL-J1-225) was grown in tryptic soy broth supplemented with 0.6% yeast extract (YE-Difco). Stock cultures of all organisms were kept at -80°C in 25% glycerol. Working cultures were streaked on either trypic soy agar (TSA, *Salmonella*), Luria Bertani agar (LBA) plates (*E. coli* O157:H7), or tryptic soy agar with 0.6% yeast extract (*L.monocytogenes*) wrapped in

parafilm and stored at 4°C for 4 weeks. For experimental purposes, all organisms were grown overnight and OD_{600} adjusted to 0.1 ($\approx 10^8$ CFU/mL) prior to experimentation.

6.3.3 Determination of minimum inhibitory concentration (MIC).

The MIC was determined by plate enumeration TSB (*Salmonella*) LB (*E. coli* O157:H7), TSB-YE (*L.monocytogenes*). Working solutions of the antimicrobial or surfactant combination were prepared by diluting the 1% stock solutions in TSB, LB, or TSB-YE to produce final LAE concentrations of 40-110 ppm. An overnight sample of the bacterial culture was diluted to approximately 10^8 CFU/ml in TSB or LB in phosphate buffered saline (8g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 800 ml of distilled H20. Adjust the pH to 7.4 with HCl. Add H_2O to 1L. Autoclave for 20mins. (Molecular Cloning)) and 1% inoculum transferred to test tubes, produce an initial cell concentration of approximately 10^6 CFU/ml. After exposure for 30s, 24hr, 48hr, 72hr, and 96hr, 50µl samples were removed from test tubes, diluted in PBS, and plated on TSA, LB agar or TSA-YE using AUTOPLATE spiral plater (Advanced Instruments INC., Norwood, MA). After incubation at 32°C (*Salmonella*, *L.monocytogenes*) or 37°C (*E. coli* O157:H7) for 48h, colonies were counted using the SCAN 500 (Interscience, France). The MIC was defined as the lowest concentration of antimicrobial agent required to inhibit development of visible growth after 24hr of incubation. The minimum detection limit was 2.0×10^1 CFU/ml.

6.3.4 Micelle composition.

To determine the most stable mixed micelle system, stock solutions of 0.25% (2500 ppm) LAE were combined with varying concentrations of *Tween-80*. After 24 hours at room

temperature, results indicated that 0.25% LAE in combination with 0.5% (5,000 ppm) *Tween-80* creates the most physically stable mixed micelle system; indicated by no sedimentation, creaming, or precipitation.

6.3.5 Membrane Potential.

Disruption of membrane potential was measured following the protocol provided with the BacLight™ Bacterial Membrane Potential Kit (Molecular Probes, Eugene, OR.). Bacteria used for analysis was grown to mid-log phase, and concentrated 10x in PBS filtered through a 0.22 µM filter prior to experimentation. Briefly, samples were treated (at the previously determined MIC) with either LAE, LAE:Tween-80, or Tween-80 for 30s, 1hr, or 3hr at room temperature. Prior to analysis, 10µL of 3mM of 3,3'-diethyloxacarbocyanine iodide ($DiOC_2(3)$) in DMSO) was added to each sample, vortexed and incubated at room temperature for 30minutes.10µL of 500 µM CCCP and 10µL $DiOC_2(3)$ were added to control samples in order to identify potential depolarization. Flow cytometry analyses were performed with a BD™ LSR II flow cytometer SORP using filters for green fluorescence of 530/30 and red fluorescence of 610/20. Each run was set to perform 10,000 events and plotted with log settings. PBS served as the sheath fluid. BD FACSDiva™ software was used for recording flow cytometry results, and analysis of data was conducted using FlowJo v10 (TreeStar Data Analysis Software).

6.3.6 Statistical analysis:

Statistical analysis was performed via One-way ANOVA and Tukeys test for comparison using Graph Pad Prism 5.

6.4 Results

6.4.1 Response of *L.monocytogenes* and *S.enterica* Enteritidis to antimicrobial exposure.

Previous experiments were conducted to determine the minimum inhibitory concentrations (MICs) of LAE, and LAE: Tween-80. Studies investigating antimicrobial properties of Tween-80 were performed, and were found to not have any bactericidal effects when used alone (data not shown). All MIC values are listed in Table 6.1 and inhibition of *L.monocytogenes* was observed at 18 and 40 ppm LAE, LAE:Tween-80 respectively while *S.enterica* Enteritidis required nearly double the concentration and inhibition was determined to be 50ppm LAE, and 100ppm LAE:Tween-80.

6.4.2 Flow cytometry based measurement of the membrane potential

Figures 6.1-6.4 detail the stability of membrane potential in *L.monocytogenes* and *S. enterica* Enteritidis after treatment with LAE, LAE:-Tween-80, or Tween-80 assessed via flow cytometry. Exposure of bacteria to proton ionophores such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) will result in a reduction of the MP to zero, indicating membrane damage (characteristic of ruptures, holes, etc.) (55). CCCP causes depolarization, and results in a shift to the left upon flow analysis while shifts to the right are an indication of hyperpolarization (K^+ leakage).Valinomycin has a high affinity for K^+, and served as a control in our experiments. Because we are interested in the effects of LAE on MP, fluorescent intensity of Valinomycin combined with each antimicrobial was used as a control, and similarities in fluorescence after antimicrobial treatment alone were compared. (Figs 6.1, 6.3)

When the Gram positive organism *L.monocytogenes* was treated with antimicrobial and compared to control samples containing 10uM Valinomycin shifts to the right were observed for treatments with LAE, LAE:Tween-80, indicating membrane disruption, but no effects were observed inTween-80 (Fig 6.1). When compared to the depolarized control nigericin, hyperpolarization was observed after exposure to all treatments. (Fig 6.2). Observing hyperpolarization after antimicrobial exposure leads us to conclude that LAE is in fact causing disrupting of MP indicated by K^+ leakage in *L.monocytogenes*.

Consistent with results obtained from *L.monocytogenes*, hyperpolarization after exposure to LAE and LAE:Tween-80 in the Gram negative organism *S. enterica* Enteritidis was observed when compared to Valinomycin (Fig 6.3). Interestingly, an early onset of potential depolarization was observed when *S. enterica* was compared to nigericin (Fig 6.4). In studies performed by Ultee *et al*, nigericin was used to diminish the pH gradient across the cytoplasmic membrane, and these findings suggest not only a disruption in membrane integrity but also a disruption in the ability to maintain a proper pH gradient.

Statistical analysis indicates that when antimicrobial treatments were applied to the Gram positive organism a significant shift in mean fluorescent intensity was observed indicating disruption in membrane potential (Fig 6.5). When applied to the Gram negative organism, no statistical significance was observed resulting in the belief that the presence of an outer membrane presents difficulties in the antimicrobial penetrating the cell (Fig 6.6).

6.5 Discussion

The stability of the membrane potential (MP) is vital in maintaining a properly functioning bacterial cell, and previous studies have associated MP with having an active

role in the generation of ATP as well as connections to bacterial auto lysis, glucose transport, chemotaxis and bacterial survival at low pH (54). Throughout this study, changes in MP after treatment with LAE, LAE:Tween-80, or Tween-80 on cellular activity and metabolic stability in *L.monocytogenes* and *S. enterica* Enteritidis was investigated.

The growth and reproduction of healthy bacterial cells is dependent upon the proper functioning of transmembrane concentration gradients (13). All cells require energy to grow and multiply, and studies dating back to the 1980s have investigated the behavior of mitochondria after exposure to lipophilic cations (48). The aim of the work presented here was to investigate the mechanistic targets after treatment with the cationic antimicrobial LAE, LAE:Tween-80, and Tween-80 at predetermined minimum inhibitory concentrations. Two ionophore's possessing affinity for K^+ was used to investigate and identify any disruption in membrane potential and/or pH gradient.

Hyperpolarization observed in cells after treatment with specific chemicals is an indication that membrane potential has been disrupted. It is a suggestion that a change in a cells membrane potential has occurred and causes the potential to become more negative. In order to combat depolarization, an efflux of K^+ will follow resulting in hyperpolarization. Porter *et al* investigated the ability of Valinomycin to induce hyperpolarization in cells after exposure, and measured changes via flow cytometry (57). Consistent with their findings, we observed a shift in fluorescence when compared to a nontreated control after 30 seconds, one hour, or three hours of exposure to LAE, and LAE: Tween-80, but not Tween-80 alone emphasizing the notion that LAE is responsible for targeting and disrupting the MP in Gram positive organisms.

Both Gram negative and Gram positive organisms were tested throughout our studies, and the most important characteristic used to distinguish the two is the structure of the bacterial cell wall. The outer membrane, specific to Gram negative organisms, allows for heightened resistance to antimicrobials due to the difficulty of penetration. Previous studies have shown that when chelators such as EDTA are added in conjunction with antimicrobials, susceptibility heightens, and when incorporated into our delivery systems a decrease in MIC values by nearly half was observed.

The findings presented throughout this work have supported the idea that although LAE does not cause cellular lysis it is effective at targeting and disrupting the membrane potential of both *L.monocytogenes* and *S. enterica.*

Table 4.1 [1]Bacterial strains were inoculated with varying amounts of LAE alone, or LAE:Tween-80 (mixed micelle), incubated at 32°C or 37°C, and samples taken every 24 hours for 96 hours. Cell survival was determined by plate enumeration.

Bacterium	Isolate	LAE MIC[1] (ppm)	LAE:Tween-80 MIC[1] (ppm)
Gram negative			
Salmonella enterica serovar Enteritidis	ATCC BAA-708	50	100
Gram positive			
Listeria monocytogenes (LM 21-Scott A)	CU FSL-J1-225	18	40

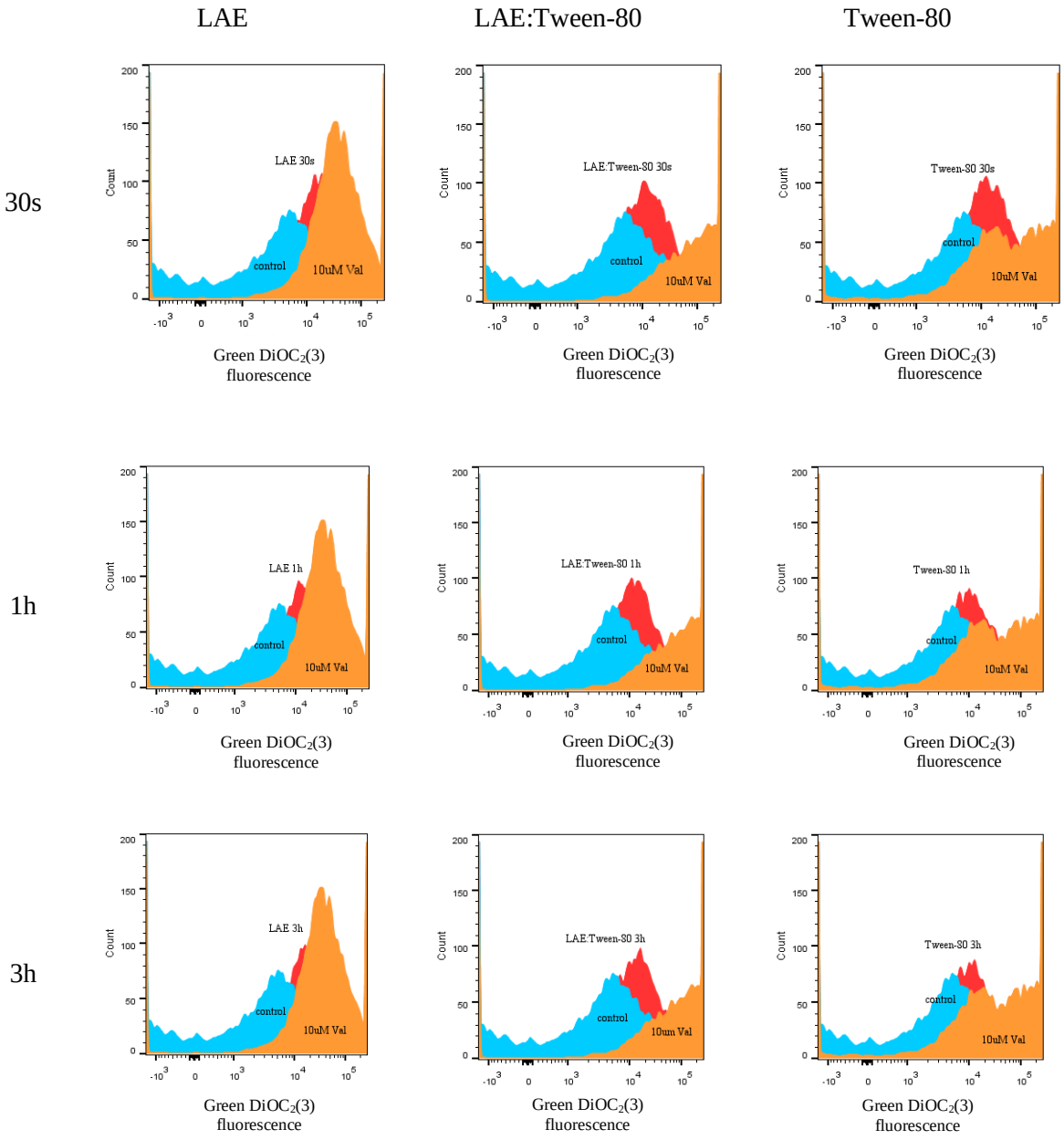


Figure 6. 1 Effects of A)18ppm LAE, B)40ppm LAE:Tween-80, or C) 80ppm Tween-80 on MP after 30s, 1h, or 3h of exposure on *L.monocytogenes* Scott A. The shift to the right after treatment with antimicrobial and compared to control with 10uM Valinomycin incubated for 1h with antimicrobial indicates hyperpolarization in membrane potential (flux of potassium ions) indicating a disruption in the membrane potential. Control samples are untreated cells stained with $DiOC_2(3)$.

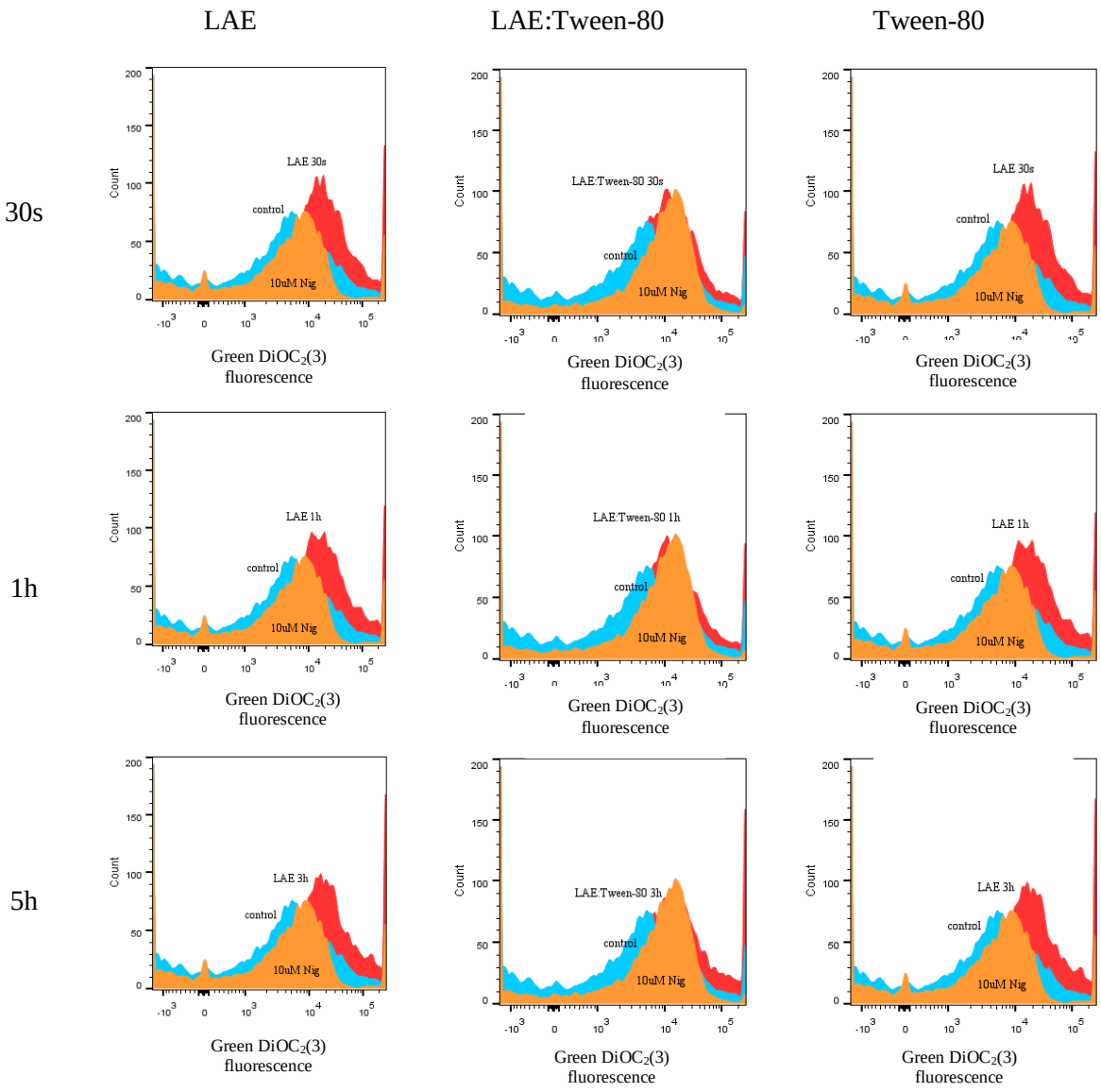


Figure 6. 2 Effects of A)18ppm LAE, B)40ppm LAE:Tween-80, or C) 80ppm Tween-80 on MP after 30s, 1h, or 3h of exposure on *L.monocytogenes* Scott A. The shift to the right after treatment with antimicrobial and compared to control with 10uM Nigericin incubated for 1h with antimicrobial indicates hyperpolarization in membrane potential (flux of potassium ions) indicating a disruption in the membrane potential. Control samples are untreated cells stained with $DiOC_2(3)$.

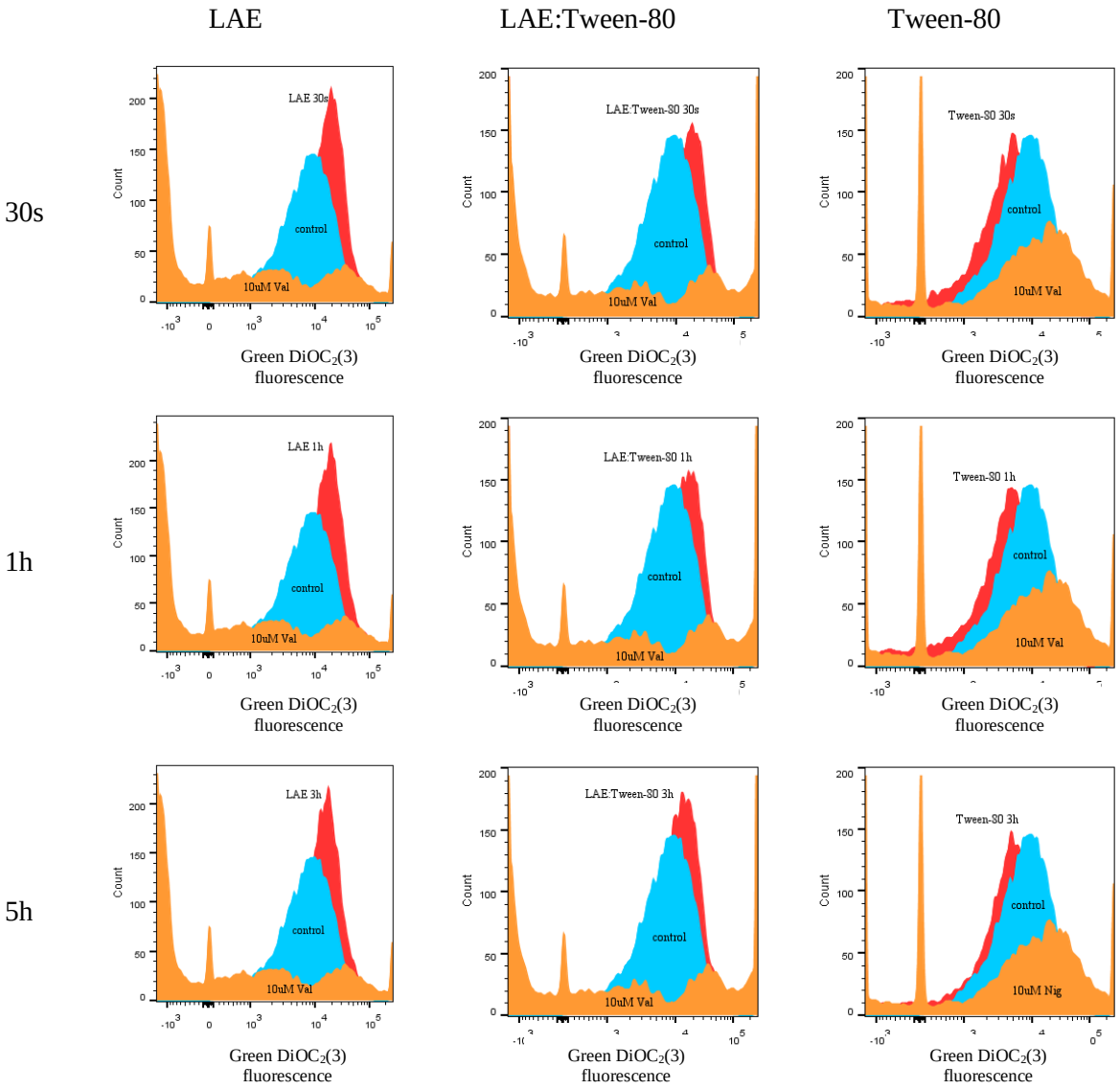


Figure 6. 3 Effects of A)50ppm LAE, B)100ppm LAE:Tween-80, or C) 200ppm Tween-80 on MP after 30s, 1h, or 3h of exposure on *S. enterica* Enteritidis. The shift to the right after treatment with antimicrobial and compared to control with 10uM Valinomycin incubated for 1h with antimicrobial indicates hyperpolarization in membrane potential (flux of potassium ions) when treated with LAE and LAE:Tween-80 indicating a disruption in membrane potential. Cells treated with Tween-80 show a shift to the left which is an indication of depolarization and disruption of the pH gradient. Control samples are untreated cells stained with $DiOC_2(3)$.

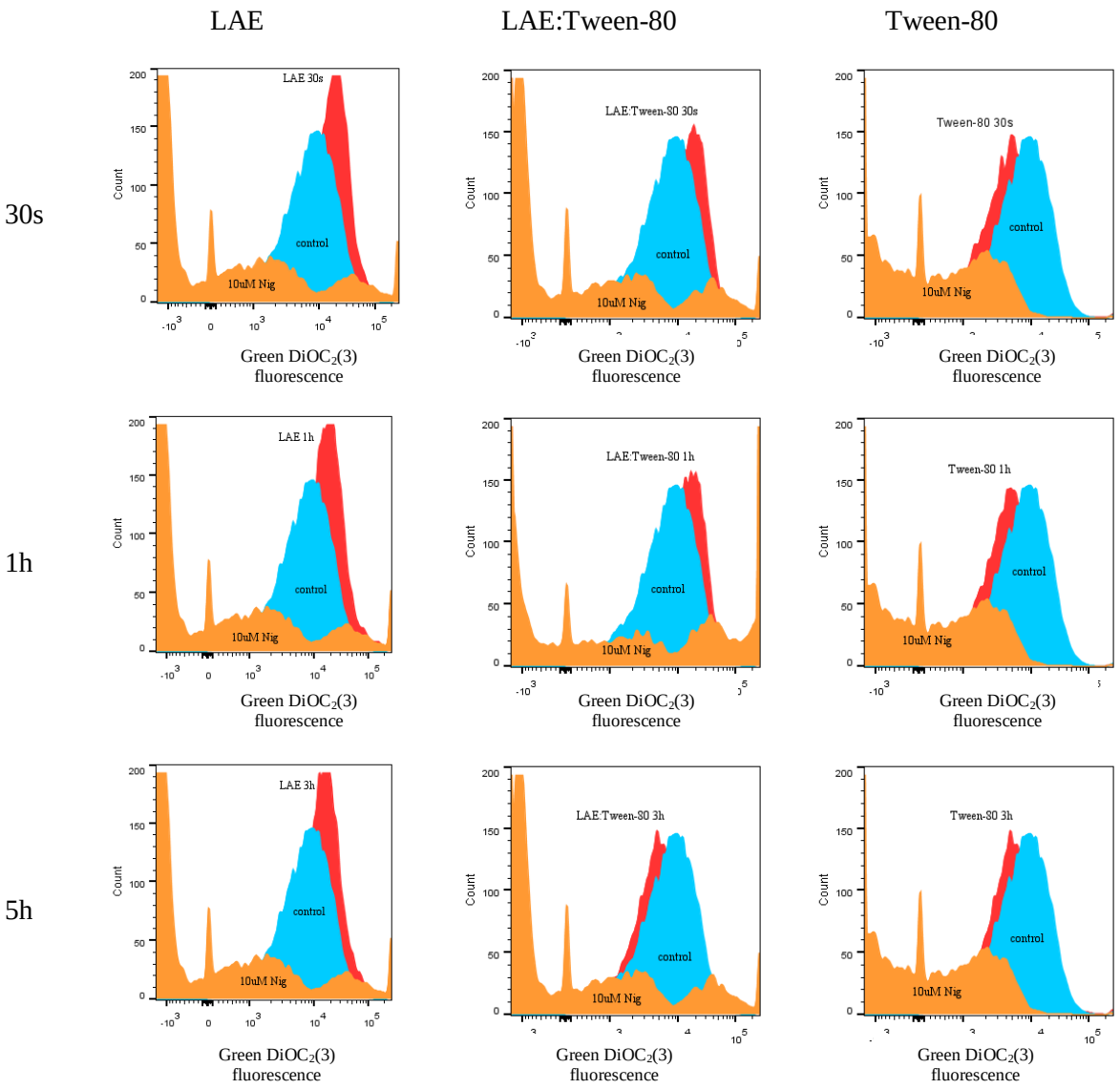


Figure 6. 4 Effects of A)50ppm LAE, B)100ppm LAE:Tween-80, or C) 200ppm Tween-80 on MP after 30s, 1h, or 3h of exposure on *S. enterica* Enteritidis. The shift to the right after treatment with antimicrobial and compared to control with 10uM Nigericin incubated for 1h with antimicrobial indicates hyperpolarization in membrane potential (flux of potassium ions) when treated with LAE and LAE:Tween-80. No changes were observed in cells treated with Tween-80 indicating a disruption in membrane potential. Cells treated with Tween-80 show a shift to the left which is an indication of depolarization and disruption of the pH gradient. Control samples are untreated cells stained with $DiOC_2(3)$.

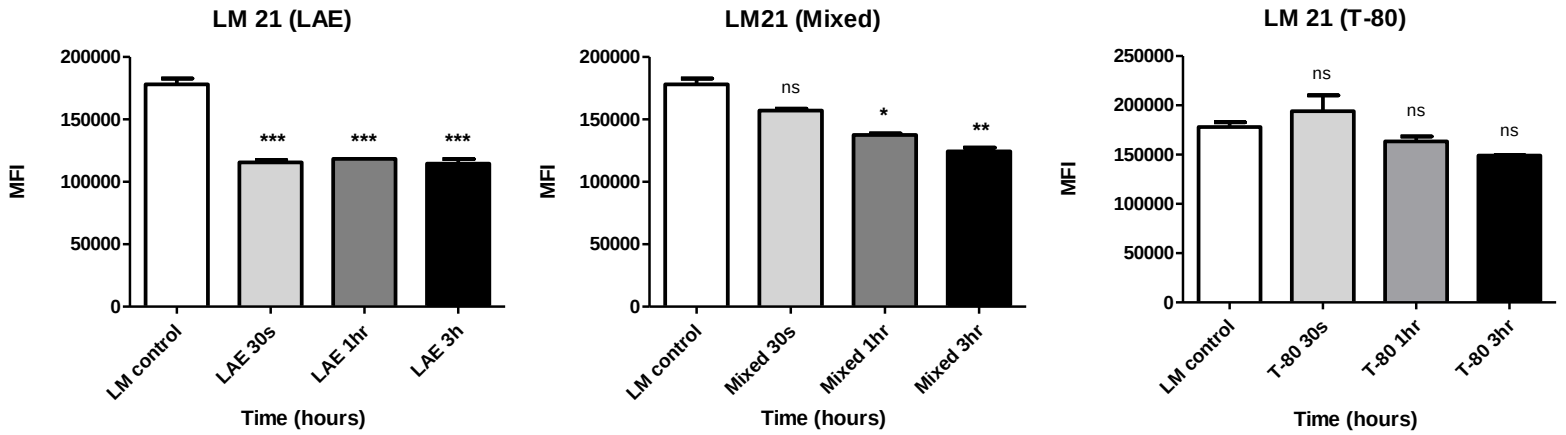


Figure 6. 5 Damage due to membrane potential of *L. monocytogenes* Scott A was measured via flow cytometry. Figure is representative of two experiments using cells grown to exponential phase and exposed to antimicrobial for 30s, 1h, and 3hrs. Bar graphs represent the mean fluorescence intensity of treatment with A)LAE (18ppm) B)LAE:Tween-80 (40ppm) or C) Tween-80 (80ppm), and error bars indicate SEM (* = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$).

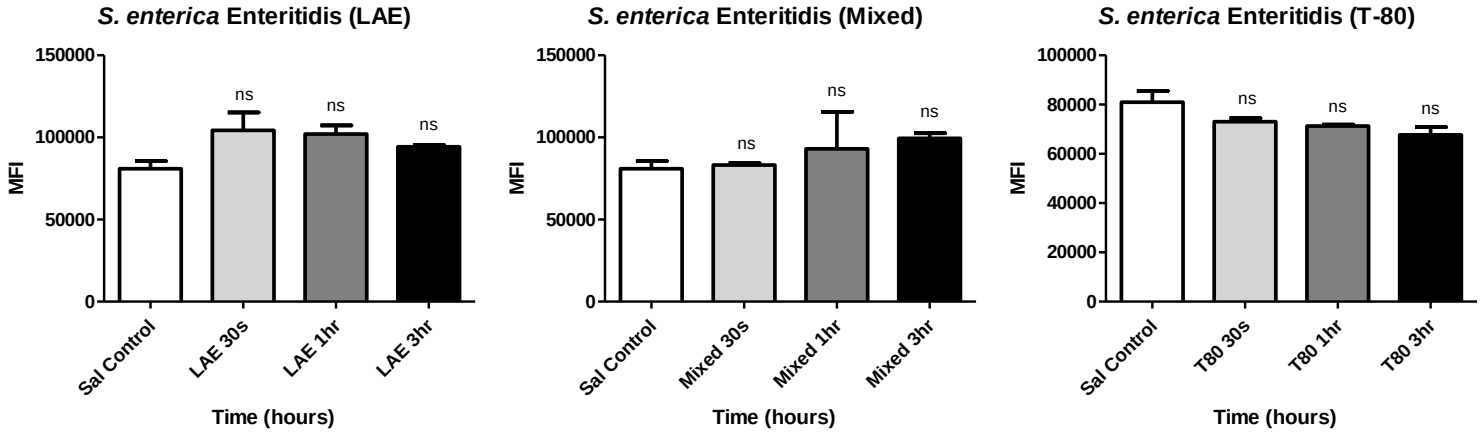


Figure 6. 6 Damage due to membrane potential of *S. enterica* Enteritidis was measured via flow cytometry. Figure is representative of two experiments using cells grown to exponential phase and exposed to antimicrobial for 30s, 1h, and 3hrs. Bar graphs represent the mean fluorescence intensity of treatment with A)LAE (50ppm) B)LAE:Tween-80 (100ppm) or C) Tween-80 (200ppm), and error bars indicate SEM. No significant difference was observed amongst treatments and control.

COMPREHENSIVE BIBLIOGRAPHY

1. **Al-Adham ISI, E. Khalil, N.D. Al-Hmoud, M. Kierans, P.J. Collier.** 2000. Microemulsions are membrane-active, antimicrobial, self-preserving systems. Journal of applied microbiology **89:**32-39.

2. **Alakomi HL.** 2003. Effect of EDTA on Salmonella enterica serovar Typhimurium involves a component not assignable to lipopolysaccharide release. Microbiology **149:**2015-2021.

3. **Alakomi HL, Paananen A, Suihko ML, Helander IM, Saarela M.** 2006. Weakening effect of cell permeabilizers on gram-negative bacteria causing biodeterioration. Appl Environ Microbiol **72:**4695-4703.

4. **Asker D, Weiss J, McClements DJ.** 2011. Formation and stabilization of antimicrobial delivery systems based on electrostatic complexes of cationic-non-ionic mixed micelles and anionic polysaccharides. J Agric Food Chem **59:**1041-1049.

5. **Asker D, Weiss, J., McClements, D.** 2008. Analysis of the interactions of a cationic surfactant (lauric arginate) with an anionic bioplymer (pectin): isothermal titration calorimetry, light scattering, and microelectrophoresis. Langmuir **25:**116-122.

6. **Aymerich T, Picouet PA, Monofort JM.** 2008. Decontamination technologies for meat products. Meat Sci **78:**114-129.

7. **Bakal G, Diaz, Alex.** 2005. The Lowdown on Lauric Arginate: Food antimicrobial hammers away at plasma membrane, disrupting a pathogen's metabolic process, Food Quality.

8. **Bonnaud M, Weiss J, McClements DJ.** 2010. Interaction of a food-grade cationic surfactant (lauric arginate) with food-grade biopolymers (pectin, carrageenan, xanthan, alginate, dextran, and chitosan). J Agric Food Chem **58:**9770-9777.

9. **Bonnaud M, Weiss, J., McClements, D.J.** 2010. Interaction of a food-grade cationic surfactant (lauric arginate) with food-grade biopolymers (pectin, carrageenan, xanhan, alginate, dextran, and chitosan). J Agr Food Chem **58:**9770-9777.

10. **Brandt AL, Castillo A, Harris KB, Keeton JT, Hardin MD, Taylor TM.** 2010. Inhibition of Listeria monocytogenes by food antimicrobials applied singly and in combination. J Food Sci **75:**M557-563.

11. **Branen JK, Davidson PM.** 2004. Enhancement of nisin, lysozyme, and monolaurin antimicrobial activities by ethylenediaminetetraacetic acid and lactoferrin. International journal of food microbiology **90:**63-74.

12. **Branen LA, Davidson PM, Salminen S, Thorngate III JH.** 2001. Food Additives, vol. 2nd Ed.

13. **Breeuwer P, Abee T.** 2004. Assessment of the membrane potential, intracellular pH and respiration of bacteria employing fluorescence techniques. Molecular Microbial Ecology Manual**:**1563-1580.

14. **Brooks JT, Sowers EG, Wells JG, Greene KD, Griffin PM, Hoekstra RM, Storockbine NA.** 2005. Non-O157 Shiga Toxin-Producing *Escherichia coli* Infections in the United States,1983-2002. J. Infect Dis **192:**1422-1429.

15. **Burt S.** 2004. Essential oils: their antibacterial properties and potential applications in foods--a review. International journal of food microbiology **94:**223-253.

16. **Cagri A, Ustunol Z, Ryser ET.** 2004. Antimicrobial edible films and coatings. Journal of Food Protection **67:**833-848.

17. **CDC** 2012, posting date. Food Safety. [Online.]

18. **CDC** 2013, posting date. National Notifiable Disease Surveillance System. [Online.]

19. **CDC.** 2013. MMWR. **62:**283-287.

20. **Chang Y, McLandsborough L, McClements DJ.** 2011. Interactions of a cationic antimicrobial (epsilon-polylysine) with an anionic biopolymer (pectin): an isothermal titration calorimetry, microelectrophoresis, and turbidity study. J Agric Food Chem **59:**5579-5588.

21. **Davidson PM, Sofos JN, Branen AL.** 2005. Antimicrobials in Food, 3rd ed. CRC Press, Boca Raton, FL.

22. **Farber JMaPIP.** 1991. *Listeria monocytogenes,* a Food-Borne Pathogen. Microbiological Reviews **55:**476-511.

23. **Freeman BA.** 1985. Burrows Textbook of Microbiology, vol. 22nd Ed. W.B. Saunders Company, Philidephia.

24. **Gaysinsky S.** 2007. Emulsions and Microemulsions as Antimicrobial Delivery Systems. University of Massachusetts Amherst.

25. **Gill AO, Holley RA.** 2004. Mechanisms of bactericidal action of cinnamaldehyde against Listeria monocytogenes and of eugenol against L. monocytogenes and Lactobacillus sakei. Appl Environ Microbiol **70:**5750-5755.

26. **Helander IM, Alakomi HL, Latva-kala K, Mattila-Sanholm T, Poi I, Smid EJ, Gorris LGM, VonWright A.** 1998. Characterization of the Action of Selected Essential Oil Components on Gram-negative bacteria. Journal of Agricultural Food Chemistry **46:**3590-3595.

27. **Hensel M, Lill H, Schmid R, Deckers-Hebestreit G, Altendorf K.** 1995. The ATP synthase (F< sub> 1</sub> F< sub> o</sub>) of< i> Streptomyces lividans</i>: sequencing of the< i> atp</i> operon and phylogenetic considerations with subunit β. Gene **152:**11-17.

28. **Holley RA, Patel D.** 2005. Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. Food Microbiology **22:**273-292.

29. **Holmberg KB, Johnson B, Kronberg B, Lindman B.** 2003. Surfactants and polymers in Aqueous Solutions, vol. 2nd ed. Wiley, New York, NY.

30. **Industrial snomechanics L** 2011, posting date. [Online.]

31. **Infante M, Pinazo A, Seguer J.** 1997. Non-conventional surfactants from amino acids and glycolipids: Structure, preparation, and properties. . Colloid Surface A **123-124:**49-70.

32. **Juneja VK, Dwivedi HP, Yan X.** 2012. Novel Natural Food Antimicrobials*. Annual Review of Food Science and Technology **3:**381-403.

33. **Kanazawa A, Ikeda T, Endo T.** 1995. A novel approach to mode of action f cationic biocides: morphological effect on antibacterial activity. Journal of Applied Bacteriology **78:**55-60.

34. **Karatzas A, Kets, E., Smid, E., Bennik, M.** 2002. The combined action of carvacrol and high hydrostatic pressure on *Listeria monocytogenes* Scott A. Journal of applied microbiology **90:**463-469.

35. **Kass EH.** 1987. A Brief Perspective on the Early History of American Infectious Disease Epidemiology. The Yale Journal of Biology and Medicine **60:**341-348.

36. **Kosswig K.** 2005. Surfactants. *In* Wiley (ed.), Ullmann's Encyclopedia of Industrial Chemistry.

37. **Kurihara K, Katsuragi Y, Matsuoka I, Kashiwayanagi M, Kumazawa T, Shoji T.** 1994. Receptor mechanisms of bitter substances. Physiol Behav. **56:**1125-1132.

38. **Law D.** 2000. The history and evoution of *Escherichia coli* O157 and other Shiga toxin-producing *E. coli*. World Journal of Microbiology & Biotechnology **16:**701-709.

39. **Lopez-de-Dicastillo C, Alonso JM, Catala R, Gavara R, Hernandez-Munoz P.** 2010a. Improving the antioxidant protection of packaged food by incorporating natural flavonoids into ethylene-vinyl-alhohol copolymer (EVOH) films. J Agr Food Chem **58:**10958.

40. **Lopez-de-Dicastillo C, Catala R, Gavara R, Hernandez-Munoz P.** 2011. Food applications of active packaging EVOH films containing cyclodextrins for the preferential scavenging of undesirable compounds. J Food Eng **104:**380-386.

41. **Lopez-de-Dicastillo C, Gallur M, Catala R, Gavara R, Hernandez-Munoz P.** 2010b. Immobilization of beta-cyclodextrin in ethylene-viny alcohol copolymer for active food packaging applications. Jounal of Membrane Science **353:**184-191.

42. **Luchansky JB, A.C.S. Porto, F.M. Wallace, and J.E. Call.** 2002. Recovery of *isteria monocytogenes* from vacuum-sealed packages of frankfurter: comparison of the U.S. Department of Agriculture (USDA) Food Safety and Inspection Service product composite enrichment method, the USDA Agricultural Research Service (ARS) product composite rinse method, and the USDA-ARS packag rinse method. J. Food Prot. **65:**567.

43. **Luchansky JB, Call JE, Hristova B, Rumery L, Yoder L, Oser A.** 2005. Viability of *Listeria monocytogenes* on commercially - prepared hams surface treated with acidic calcium sulfte and lauric arginate and stored at 4^0C. Meat Sci **71:**92-99.

44. **Luchansky JB, J.E. Call, B. Hristova, L. Rumery, . Yoder, and A. Oser.** 2005. Viability of *Listeria monocytogenes* on commercially prepared hams surface treated with acidic calcium sulfate and lauric arginate and stored at 4^0C. *Meat Sci* **71:**92-99.

45. **Ma Q, Davidson PM, Zhong Q.** 2013. Antimicrobial properties of lauric arginate alone or in combination with essential oils in tryptic soy broth and 2% reduced fat milk. International journal of food microbiology **166:**77-84.

46. **Ma Q, Davidson, Michael, Zhong Qixin.** 2013. Antimicrobial properties of lauric arginate alone or in combination with essential oils in tryptic soy broth and 2% reduced fat milk. International journal of food microbiology **166:**77-84.

47. **Marcos B, Aymerich T, Monofort JM, Garriga M.** 2007. Use of antimicrobial biodegradeable packaging to control *Listeria monocytogenes* during storage of cooked ham. International journal of food microbiology **120:**152-158.

48. **Marques-Santos LF, Coqueiro VM, Rumjanek VM.** 2006. Cyclosporin A does not protect the disruption of the inner mitochondrial membrane potential induced by potassium ionophores in intact K562 cells. Cell biology international **30:**197-204.

49. **Mason DJ, Gant VA** 2013, posting date. Flow Cytometry and Microbiology. [Online.]

50. **McCabe-Sellers BJ, Beattie SE.** 2004. Food safety: emerging trends in foodborne illness surveillance and prevention. Journal of the American Dietetic Association **104:**1708-1717.

51. **McClements DJ.** 2007. Critical Review of Technique nd Methodologies for Characterization of Emulsion Stability. Crit Rev Food Sci **47:**611-649.

52. **McClements DJ.** 2005. Food emulsions, principles, practices, and techniques. CRC Press, Boca Raton, FL.

53. **Muriel-Galet V, Lopez-Carballo G, Gavara R, Hernandez-Munoz P.** 2012. Antimicrobial food packaging film based on the release of LAE from EVOH. International journal of food microbiology **157:**239-244.

54. **Neumeyer A, Hübschmann T, Müller S, Frunzke J.** 2013. Monitoring of population dynamics of Corynebacterium glutamicum by multiparameter flow cytometry. Microbial biotechnology **6:**157-167.

55. **Novo DJ, Perlmutter NG, Hunt RH, Shapiro HM.** 2000. Multiparameter Flow Cytometric Analysis of Antibiotic Effects on Membrane Potential, Membrane Permeability, and Bacterial Counts of Staphylococcus aureus andMicrococcus luteus. Antimicrobial agents and chemotherapy **44:**827-834.

56. **Paulsen IT, Brown MH, Ronald AS.** 1996. Proton-dependent multidrug systems. Microbiological Reviews **42:**575-608.

57. **Porter J, Pickup R, Edwards C.** 1995. Membrane hyperpolarisation by valinomycin and its limitations for bacterial viability assessment using rhodamine 123 and flow cytometry. FEMS microbiology letters **132:**259-262.

58. **Rodriguez E.** 2004. Cellular effects of monohydrochloride of L-arginine, Nalpha-lauroyl ethylester (LAE) exposure on *Salmonella typhimurium* and *Staphylococcus aureus*. Journal of applied microbiology **96:**903-912.

59. **Rosen MJ.** 2004. Surfactants and Interfacial Phenomena 3rd ed., p. 444, 3rd ed. John Wiley & Sons, Inc., Hoboken, New Jersey.

60. **Sewald M, Jon DeVries.** Food Product Shelf LIfe. *In* Laboratories M (ed.), Medallion Laboratories.

61. **Shelef L, Jyothi, E., Bulgarellii, M.** 2006. Growth of enteropathogenic and spoilage bacteria in sage-containing broth and foods. J Food Sci **49:**737-740.

62. **Soni K, Nannapaneni, R., Schilling, M., Jackson, V.** 2010. Bactericidal activity of lauric arginate in milk and Queo Fresco cheese against *Listeria monocytogenes* cold growth. Journal of Dairy Science **93:**4518-4525.

63. **Stopforth JD, Visser, D., Zumbrink, R, Van Dijk, L, and Bontenbal, E.W.** 2010. Control of Literia monocytogenes on Cooked Cured Ham by Formulation with a Lactate-Diacetate Blend and Surface Treatment with Lauric Arginate. Journal of Food Protection **73:**552-555.

64. **Suller MTE, Russel AD.** 2000. Triclosan and antibiotic resistance in *Staphylococcus aureus*. Journal of Antimicrobial Chemotherapy **46:**11-18.

65. **Suppakul P, Milts J, Sonneveld K, Bigger SW.** 2003. Active packaging technologies with an emphasis on antimicrobial packaging and its applications. J Food Sci **68:**408-420.

66. **Taormina PJ, Doras, W.J.** 2009. Short-Term Bactericidal Efficacy of Lauric Arginate against *Listeria monocytogenes* Present on the Surface of Frankfurters. *J Food Prot* **72:**1216-1224.

67. **Tattawasart U, Hann AC, Maillard JY, Furr JR, Russel DD.** 2000. Cytological changes in chlorhexidine-resistant isolated of *Pseudomonas stutzeri*. Journal of Antimicrobial Chemotherapy **45:**145-152

68. **Tauxe R.** 2002. Emerging foodborne pathogens. International journal of food microbiology **78:**31-41.

69. **Theinsathid P, Visessanguan W, Kruenate J, Kingcha Y, Keeratipibul S.** 2012. Antimicrobial activity of lauric arginate-coated polylactic acid films against *Listeria monocytogenes* and *Salmonella typhimurium* on cooked sliced ham. J Food Sci **77:**145-152.

70. **Toutain-Kidd CM, Kadivar SC, Bramante CT, Bobin SA, Zegans ME.** 2009. Polysorbate 80 inhibition of Pseudomonas aeruginosa biofilm formation and its cleavage by the secreted lipase LipA. Antimicrobial agents and chemotherapy **53:**136-145.

71. **Ultee A, Bennik MHJ, Moezelaar R.** 2002. The Phenolic Hydroxyl Group of Carvacrol Is Essential for Action against the Food-Borne Pathogen Bacillus cereus. Applied and Environmental Microbiology **68:**1561-1568.

72. **US FDA** Oct 25, 2013 2013, posting date. Food Safety Modernization Act (FSMA). [Online.]

73. **US FDA** 2013, posting date. Foodborne Illness: What You Need to Know. [Online.]

74. **USDA** 2005, posting date. Agency Response Letter GRAS Notice No. GRN 000164. [Online.]

75. **USDA FSIS.** 2003. Control of *Listeria monocytogenes* in ready-to-eat meat and poultry products; final rule. *Fed. Regist.* **68:**34208-34252.

76. **Vazquez-Boland JA, Kuhn M, Berche P, Chakraborty T, Dominguez-Bernal G, Goebel W, Gonzalez-Zorn B, Wehland J, Kreft J.** 2001. Listeria pathogenesis and molecular virulence determinants. Clinical microbiology reviews **14:**584-640.

77. **WHO.** 2009. Safety evaluation of certain food additives. WHO food additives series **60:**39-84.

78. **Willey JM, Sherwood LM, Woolverton CJ.** 2008. Prescott, Harley, and Klein's Microbiology, 7th ed. McGraw-Hill, New York, NY.

79. **Woodcock N, Hammond, B., Ralyea, R., Boor, K.** 2009. Short communication: N [alpha]-lauroyl-L-arginine ethylester monohydrochloride reduces bacterial growth in pasteruized milk. Journal of Dairy Science **92:**4207-4210.

80. **Zhang L, Rozek A, Hancock RE.** 2001. Interaction of cationic antimicrobial peptides with model membranes. The Journal of biological chemistry **276:**35714-35722.

81. **Zhang Y, Yam K, Chikindas M.** 2004. Effective control of *Listeria monocytogenes* by combination of nisin formulated and slowly released into a broth system. International journal of food microbiology**:**15-22.

82. **Ziani K, Chang Y, McLandsborough L, McClements DJ.** 2011. Influence of surfactant charge on antimicrobial efficacy of surfactant-stabilized thyme oil nanoemulsions. J Agric Food Chem **59:**6247-6255.